NUTRITIONAL STATUS AND INFLAMMATION IN INFANTS AND YOUNG CHILDREN WITH CYSTIC FIBROSIS

By

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NUTRITIONAL STATUS AND INFLAMMATION IN INFANTS AND YOUNG CHILDREN WITH CYSTIC FIBROSIS

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Cystic fibrosis (CF) is one of the most common autosomal recessive genetic conditions in the Caucasian population. CF is a life-threatening disease and involves multiple organ systems. CF is caused by a mutation in the gene that encodes for the cystic fibrosis transmembrane conductance regulator (CFTR) protein and dysregulation of CFTR results in the formation of a thick mucus layer in the lungs, intestines, and pancreatic ducts that lead to nutritional deficiencies and poor lung outcomes. The objectives of this dissertation are to examine the nutritional status, mainly vitamin D and essential fatty acid (EFA) status, and the relationship with inflammation in a multicenter, prospective, longitudinal, observational trial (FIRST study) in infants and young children with CF in the first 2 years of life.

Our study evaluated the effectiveness of current recommended vitamin D supplementation doses in achieving optimal vitamin D status, i.e. 25-hydroxyvitamin D [25(OH)D] ≥30 ng/mL and found that they are effective in the majority of our cohort, leading to a significantly lower prevalence of vitamin D insufficiency in our cohort compared to otherwise healthy children. We have also reported a high prevalence of EFA abnormalities in our cohort (31% at 4 months, 35% at 12 months, and 48% at 24 months) in the absence of symptoms of EFA deficiency, prompting the need to monitor EFA routinely in practice. Our study also showed inverse relationships between pro-inflammatory markers and n-6 fatty acids (linoleic

acid and arachidonic acid) which is suggestive that a higher linoleic acid intake may ameliorate EFA abnormalities in CF.

To explain variation in vitamin D responses in our cohort, we are recruiting for a whole genome sequencing sub-study and will investigate the variants associated with low 25(OH)D and determine how much these variants contribute to the variation in responses that was observed. Future research on EFA supplementation is warranted to further understand the mechanism of fatty acid metabolism in CF and its association with inflammation.

In summary, these data demonstrated that current guidelines for management of vitamin D status is effective and pushes for the need to monitor EFA status routinely in clinical practice.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF AUTHOR-DEFINED ABBREVIATIONS

25(OH)D	25-hydroxyvitamin D
AA	Arachidonic acid
AI	Adequate intake
ALA	Alpha-linolenic acid
BAL	Bronchoalveolar lavage fluid
CF	Cystic fibrosis
CFF	Cystic Fibrosis Foundation
CFTR	Cystic fibrosis transmembrane conductance regulator
CRP	C-reactive protein
DBP	Vitamin D binding protein
DHA	Docosahexaenoic acid
DGLA	Dihommo-gammalinolenic acid
DXA	Dual energy x-ray absorptiometry
EAR	Estimated average requirement
EFA	Essential fatty acid
EFAD	Essential fatty acid deficiency
EFAI	Essential fatty acid insufficiency
EFAS	Essential fatty acid sufficiency
EPA	Eicosapentaenoic acid
ER	Early responder
FEV_1	Forced expiratory volume in 1 second
FIRST	Feeding Infants Right from the Start

GLA	Gamma-linolenic acid
HETE	Hydroxyeicosatetraenoic acids
HPLC	High-performance liquid chromatography
IL	Interleukin
IRT	Immunoreactive trypsinogen
LA	Linoleic acid
LR	Late responder
LT	Leukotriene
MI	Meconium ileus
NBS	Newborn screening
NR	Non-responder
NHANES	National Health and Nutrition Examination Survey
PERT	Pancreatic enzyme replacement therapy
PG	Prostaglandin
PI	Pancreatic insufficiency
PS	Pancreatic sufficiency
PUFA	Polyunsaturated fatty acids
RBC	Red blood cells
RDA	Recommended dietary allowance
TNF-α	Tumor necrosis factor-α
TR	Transient responder
T:T	Triene-to-tetraene ratio
TX	Thromboxane

VDR	Vitamin D receptor
VDRE	Vitamin D response element
VDSP	Vitamin D standardization program
WGS	Whole genome sequencing

CHAPTER 1

REVIEW OF LITERATURE

CYSTIC FIBROSIS

Overview of Cystic Fibrosis

Cystic fibrosis (CF) is one of the most common autosomal recessive genetic diseases in the Caucasian population with an estimated incidence of approximately 1 in 3000 live births (1). Around 30,000 individuals in the US have CF according to the latest patient registry data from the CF Foundation (CFF) (2). CF was first recognized as a separate clinical entity in 1938 and was initially described as the fibrosis of the pancreas (3). However, in 1989, it was discovered that CF is caused by a mutation in the gene that encodes for the cystic fibrosis transmembrane conductance regulator (CFTR) protein (4). Cystic fibrosis is generally characterized by elevated chloride concentrations in sweat as a result of dysfunctional sodium and chloride transport in cells, pancreatic insufficiency (PI), and progressive pulmonary disease (5, 6).

Pathogenesis and Clinical Manifestations of Cystic Fibrosis

CF is a progressive disease that affects the lungs, digestive system, sweat glands, and reproductive tract (6). The CFTR gene encodes for the CFTR protein, which is a protein kinase A-activated, ATP-gated anion channel located primarily in the apical membrane of epithelial cells of the gastrointestinal tract, the airway, the biliary duct, the sweat ducts, the pancreas, and parts of the reproductive organs (7, 8). The CFTR protein functions mainly as a chloride channel to regulate the flow of chloride (9, 10). In tissues expressing CFTR, fluid secretion is primarily controlled by the extent of transcellular chloride transport, with the rate of chloride exiting across the apical membrane as the rate-limiting step (11, 12). Additionally, CFTR protein regulates the epithelial sodium channel and ATP channels, inhibits the calcium-activated chloride channels, and is involved in the bicarbonate-chloride exchange.

In CF, dysregulation of CFTR protein leads to increased fluid and sodium resorption in the respiratory tract, thus forming a viscous airway surface liquid layer (13, 14). CFTR dysfunction also leads to impaired bicarbonate transport and altered secretions in the pancreas, which leads to mucoviscidosis and blockages in the pancreatic ducts (15-19).

Meconium ileus (MI) is the most dramatic onset of CF and happens shortly after birth where the newborns have an intestinal obstruction with dehydrated meconium, which can lead to intestinal resection. MI occurs in about 20% of newborns diagnosed with CF. Infants with MI have been found to poorer nutritional status and growth compared to other patients with CF without MI (20). Among patients with CF, about 85% will develop PI due to blocked pancreatic ducts and reduced secretion of pancreatic enzymes (21). Additionally, patients with CF have lungs that appear normal in appearance at birth but are quickly infected and inflamed even during infancy when they are asymptomatic (22). These infections can become chronic as the child grows and can eventually lead to bronchiectasis and respiratory failure (13).

More than 2000 mutations have been identified to date (www.genet.sickkids.on.ca/cftr/); the most common mutation is a 3 base pair deletion at the amino acid position 508 of the CFTR gene that leads to the loss of a phenylalanine residue, otherwise known as F508del (2, 23, 24). The mutations are classified based on their effect on CFTR protein production, trafficking, function, or stability (25). Class I mutations are mutations that lead to no functional CFTR protein as a result of the presence of premature stop codons. Class II mutations, also the most common mutation class, lead to protein misfolding and prevent the trafficking of CFTR protein to the apical membrane. Approximately 50% of individuals with CF are homozygous for F508del and another 40% are F508del heterozygotes (26). Class III mutations, also known as gating mutations, lead to reduced or lack of opening of the CFTR protein on the apical

membrane. Class IV mutations lead to decreased channel conductance, i.e. decreased flow of ions out of the cell. Class V mutations lead to reduced CFTR synthesis and thus few functional CFTR proteins are present on the apical membrane. Lastly, class VI mutations cause reduced CFTR stability on the apical membrane as there is an increase in CFTR turnover at the cell surface (25). Severe CFTR mutations, i.e. classes I to III, are associated with dramatically decreased pancreatic ductal flow and absent digestive enzymes (8, 27). The other classes of mutations are generally associated with pancreatic sufficiency (PS) (28).

Diagnosis and Treatment of Cystic Fibrosis

Nationwide newborn screening (NBS) for CF became available in 2010 and created unprecedented opportunities for clinical interventions and research in infants with CF. Dried blood spot screening for CF in newborns was first described in 1979 (29). A positive CF screening is based on initial detection of elevated immunoreactive trypsinogen (IRT) (29, 30); the second tier of the screening is genetic analysis to identify CFTR mutations (31, 32). Once the infant has a positive NBS for CF, sweat chloride testing should be performed as soon as possible to prevent delay in diagnosis. Sweat chloride testing is the gold standard to confirm CF if the sweat chloride value is greater than or equal to 60 mmol/L in infants with a positive NBS (33, 34). It is important to understand that a positive IRT test alone is not equivalent to a diagnosis of CF. In fact, only about 1% of newborns with positive IRT have CF. Moreover, about 5% of individuals with CF had a false negative IRT screen at birth, so not all cases are detected by screening for elevated IRT (35).

There is strong evidence that NBS for CF has provided earlier opportunities to prevent malnutrition in infants with CF (36-39). Generally, CF is associated with an increased risk of

protein-energy malnutrition and deficiencies in fat-soluble vitamins and essential fatty acids. As such, poor growth, abnormalities in biochemical indices of nutritional status, and clinical symptoms of malnutrition have been historically reported in individuals with CF. Studies have shown that earlier diagnosis and intervention of infants with CF have led to early growth recovery which is associated with better nutritional and pulmonary outcomes, and hence resulting in improved child survival and sustained growth benefits through puberty (38-45).

Without therapy, CF is usually fatal within the first few years of life. Current treatment is multifaceted and requires close monitoring by an expert multidisciplinary care team. Traditionally, treatment of CF involved the use of airway clearance therapy, antibiotics for infections, inhaled therapies for reducing the frequency of pulmonary exacerbations, bronchodilators to increase air flow to the lungs, pancreatic enzyme supplementation to improve nutrient absorption, and fat-soluble vitamin supplementation to prevent deficiencies (6, 46-52) . In recent years, CFTR modulators have been developed and proven to be efficacious in improving the function of mutant CFTR proteins. CFTR modulators can work as potentiators, which improve the probability of the opening of the CFTR protein, correctors, which enhance trafficking of CFTR processing variants to apical membrane, or stabilizers, which increase the stability of the CFTR protein on the membrane (25, 53-55). There are currently 3 FDA-approved drugs, namely ivacaftor (Kalydeco®), lumacaftor/ivacaftor (Orkambi®), and tezacaftor/ivacaftor (Symdeko®), available on the market for individuals with CF. These drugs target individuals with different CFTR mutations; for example, Kalydeco® is now approved for children aged 6 months and older for individuals with at least one of the 38 approved mutations, mainly gating and alternative splicing mutations (56, 57). Orkambi® is approved for children aged 2 years and above and who are F508del homozygotes (58, 59). Symdeko® is approved for

children aged 12 years and older and are for individuals who have at least one copy of F508del mutation (60, 61).

Nutritional Management of Cystic Fibrosis

PI is present at birth in about 60% of infants with CF diagnosed through NBS, and by 1 year of age, 90% of infants with CF will have PI (21, 62). The transition from PS to PI is clinically significant because most nutrition-related issues with CF are a result of PI. Malabsorption and maldigestion caused by PI can be attributed to lack of digestive enzymes, impaired bicarbonate secretion, and loss of bile salts and bile acids as a result of dysfunctional CFTR (17). Pancreatic enzyme replacement therapy (PERT) is effective in reducing malabsorption in the CF population (48, 63-65) and is initiated as soon as the infant is known to have 2 CFTR mutations associated with PI (48). However, even with the administration of PERT, lipid malabsorption persists (66).

Some studies have observed that patients with CF had an increase in energy expenditure compared to otherwise healthy individuals (67-69), possibly due to increased work of breathing and chronic respiratory infections. One study had shown that individuals with CF can have a metabolic rate that is 25-80% higher than individuals without CF (70). As such, poor linear growth has been observed in CF clinics (71, 72); energy intakes 10% more than the estimated energy requirements with 35-40% of energy from fat are now recommended for individuals with CF (46, 47). The current guidelines recommend for children with CF to maintain a weight-for-age percentile of 50% (48). A weight-for-age percentile >10% at 4 years of age was positively associated with better pulmonary outcomes from 6 to 18 years of age (43). This study also found that subjects with a weight-for-age percentile >50% at 4 years were less likely to have impaired

glucose tolerance, fewer hospitalizations, and less likely to have CF-related diabetes at 18 years of age.

Fat-soluble vitamin deficiencies have also been observed in the CF population with varying prevalence (40, 73-79). An older, longitudinal cohort study that investigated infants diagnosed with CF via NBS reported that 46% of infants had at least one vitamin deficiency at around 2 months of age; this improved to 6% by 6 years of age. However, the cut-offs used in this study were lower than the current recommended concentrations for some biomarkers used to determine deficiencies, suggesting an underestimation of prevalence of deficiencies (48, 74). An Australian study that looked at vitamins A and E in infants diagnosed via NBS reported that about 50% of infants were vitamin A deficient and 25% were vitamin E deficient at around 2 months of age and these corrected with supplementation in all but one infant by 1 year of age (75).

Interestingly, elevated serum retinol concentrations in the CF population has been a concern in the past decade. Recent evidence suggests that current practices in vitamin A supplementation has led to elevated serum retinol concentrations (80-82). One study found that almost 60% of the adolescents with CF had elevated serum retinol concentrations; this mainly stems from vitamin A intake from CF-specific multivitamins that contain varying amounts of preformed vitamin A (81).

VITAMIN D IN CYSTIC FIBROSIS

Metabolism and Functions of Vitamin D

Vitamin D is formed when 7-dehydrocholesterol in the epidermis is converted to previtamin D_3 by UVB and then converted to cholecalciferol (vitamin D_3). Vitamin D_3 in

circulation is transported into the liver and converted into 25-hydroxyvitamin D [25(OH)D], which is the major circulating vitamin D metabolite. 25(OH)D is hydroxylated in the kidney by 1α-hydroxylase to 1,25-hydroxyvitamin D ($1,25(OH)_2D$), the most biologically active vitamin D metabolite (83, 84). Quantification of 25(OH)D is the current common practice in assessing vitamin D status (85).

The major biological function of vitamin D is to regulate blood calcium homeostasis (86). When blood calcium concentrations are low, parathyroid hormone (PTH) is released from the parathyroid glands. PTH triggers the formation of $1,25(OH)_2D$ by upregulating the expression of 1α-hydroxylase (87). $1,25(OH)_2D$ increases the absorption of dietary calcium via the intestines and stimulates the development of osteoclasts for bone resorption, resulting in an increase in blood calcium levels which inhibits PTH release.

Other functions of vitamin D have been discovered. $1,25(OH)_2D$ can bind to the vitamin D receptor (VDR) that translocates to the nucleus and binds to the vitamin D response element (VDRE) on target genes to regulate their expression (88). $1,25(OH)_2D$ also has a regulatory role in insulin secretion. In rats, vitamin D deficiency impaired glucose-mediated insulin secretion and glucose tolerance. Similarly, in humans, low serum 25(OH)D concentrations were significantly associated with insulin resistance and impaired β-cell function (89-92). Vitamin D also has several effects on the immune system. Studies have shown that suboptimal vitamin D status is associated with an increased risk of autoimmune and infectious diseases (93, 94).

Prevalence and Causes of Vitamin D Deficiency and Insufficiency in Cystic Fibrosis

The prevalence of vitamin D deficiency and insufficiency in CF varies greatly depending on the cut-off used in the studies. Reportedly, the prevalence of vitamin D deficiency and

insufficiency has been as high as 90% across all ages of individuals with CF (74, 95-99). While most studies reported vitamin D deficiency and insufficiency in subjects with PI, some studies have also reported vitamin D deficiency and insufficiency in subjects with PS, suggesting that vitamin D deficiency in CF is more than just intestinal malabsorption. One study showed that about 50% of subjects with PS were vitamin D deficient or insufficient whereas 40% of subjects with PI were vitamin D deficient or insufficient with no statistically significant differences between the group mean 25(OH)D concentrations (100).

Several factors have been proposed to contribute to vitamin D deficiency in CF (101). Seasonal variability has been reported in some studies that found differences in the prevalence of vitamin D deficiency and insufficiency among the seasons, reinforcing the suggestion to screen for vitamin D in the winter months instead of the summer months (97, 102). Reduced vitamin D binding protein (DBP) was also observed in the CF population (103) suggesting that there is less 25(OH)D in circulation since about 85% of 25(OH)D in circulation is bound to DBP (104). Low concentration of DBP is associated with low serum 25(OH)D concentrations and thus DBP has been suggested as another possible nutritional marker in the CF population (103).

Vitamin D Deficiency and its Associated Clinical Outcomes in Cystic Fibrosis

In CF, serum 25(OH)D concentrations had been found to be positively correlated with lung function as assessed by forced expiratory volume in 1 second (FEV_1) % predicted. The study found that CF patients with other co-morbidities, such as CF-related diabetes, liver disease, and mucoid *Pseudomonas aeruginosa*, had significantly lower 25(OH)D concentrations than CF patients without other co-morbidities (105). A study that looked at associations with vitamin D deficiency in children under 12 years of age found that the median 25(OH)D concentration was significantly lower in children who had *Pseudomonas* than those who were never colonized with

Pseudomonas (100). Another study also found that adolescents who were vitamin D deficient had significantly more pulmonary exacerbations than those who were vitamin D insufficient or sufficient (106). Additionally, vitamin D deficiency was identified as a risk factor for CF-related diabetes in the Scandinavian Cystic Fibrosis Nutritional Study (107). Therefore, maintaining vitamin D sufficiency is crucial for individuals with CF not only because of bone health, but also because of its associated clinical outcomes.

Evaluation of Vitamin D Status

Total serum 25(OH)D is the recommended biomarker for evaluation of vitamin D status in the CF population. 25(OH)D is not the active form of vitamin D and has a half-life of 14-21 days compared to $1,25(OH)_2D$ which is the active form of vitamin D and has a half-life of 4 hours. According to the CFF guidelines, serum $1,25(OH)_2D$ should not be used to assess vitamin D status as the concentration of $1,25(OH)_2D$ can be variable independent of vitamin D deficiency because it does not reflect endogenous vitamin D (108).

It is recommended that to assess the vitamin D status of individuals with CF, serum 25(OH)D should be measured annually and rechecked 3 months after the dose of vitamin D supplementation has been changed. Individuals with serum 25(OH)D concentration 30 ng/mL or greater are considered to have optimal vitamin D status or vitamin D sufficient, vitamin D insufficiency is defined as serum 25(OH)D concentration less than 30 ng/mL but greater than or equals to 20 ng/mL, and vitamin D deficiency is defined as serum 25(OH)D concentration less than 20 ng/mL (108).

There has been some deliberation regarding the need for serum 25(OH)D concentration to be greater than 30 ng/mL. The Institute of Medicine (109) and American Academy of

Pediatrics (110) had proposed a serum 25(OH)D concentration greater than 20 ng/mL as optimal vitamin D status for otherwise healthy children whereas The Endocrine Society (111) had recommended for serum 25(OH)D concentrations to be greater than 30 ng/mL. Yet, no studies have directly shown the benefits of setting the goal to be above 30 ng/mL versus 20 ng/mL in the CF field. Moreover, vitamin D toxicity can lead to hypercalcemia and symptoms such as nausea and vomiting; nonetheless, vitamin D toxicity is an uncommon occurrence especially in the CF field (111, 112). Given the current knowledge and lack of reliable evidence in the literature, the guidelines committee adopted the cutoff of 30 ng/mL as optimal (108). As such, the decision to increase vitamin D supplementation beyond the maximum dose when serum 25(OH)D concentration is constantly between 20 ng/mL and 30 ng/mL despite increase in supplementation is debatable.

Vitamin D Compounds and Treatment Strategies

There are 2 forms of vitamin D available for supplementation, i.e. D_2 (ergocalciferol) and D_3 (cholecalciferol). Generally, it has been established in the CF field that D_3 is the form of recommended vitamin D supplementation. A study was done previously to assess the relative efficacy of vitamin D therapy via D_2, D_3, or use of UV light. This study concluded that oral vitamin D supplementation was more efficacious than UV light therapy and that consumption of D_3 was more effective in increasing 25(OH)D concentrations than D_2 in individuals with CF (113). Similar results were also replicated in other studies which proved that D_3 was more efficacious than D_2 in increasing 25(OH)D concentrations in both the CF population and otherwise healthy population; even high doses of D_2 did not correct vitamin D deficiency in CF patients (114-116).

Dietary Sources of Vitamin D

Very few foods contain vitamin D naturally; some notable foods include fatty fish and fish liver oils. Table 1 shows the vitamin D content in selected food items. There is an insignificant amount of vitamin D in breastmilk compared to vitamin D in infant formulas; therefore, the American Academy of Pediatrics recommends exclusively and partially breastfed infants to be supplemented with 400 IU/day of vitamin D, as well as infants who are formula fed and consuming less than 1L of infant formula per day (110).

Table 1. Vitamin D content in selected food items (117, 118)

Food item	Serving size	Vitamin D (IU) per serving
Fish oil, cod liver	100g	10000
Fish, halibut	100g	1097
Fish, salmon	100g	563
Infant formula, powder	100g	300 – 380
Egg	100g	82
Yogurt, non-fat, low-fat, full-fat, vitamin D-fortified	100g	47 – 60
Milk, skim, 2%, whole, vitamin D-fortified	100mL	42 – 49
Cheese, cheddar	100g	41
Mature human milk, 2 weeks postpartum	1000mL	13.2

Dietary Reference Intakes and Recommendations from Cystic Fibrosis Foundation for Vitamin D

Table 2 shows the dietary reference intakes for vitamin D for infants and children as well as the recommendations from the CFF. There is not enough evidence to establish an estimated average requirement (EAR) for children under 1 year of age and as such, an adequate intake (AI)

was developed. The CFF recommends the same initial dose of vitamin D intake for children under 1 year of age.

In the most recent vitamin D guidelines by the CFF, a stepwise approach was included in the recommendations to increase vitamin D supplementation in response to serum 25(OH)D concentrations. The CFF recommends that from birth to 12 months of age, children with serum 25(OH)D concentration of at least 20 ng/mL but less than 30 ng/mL, and with confirmed adherence, the dose of vitamin D should be increased to step 1, i.e. 800-1000 IU/day for this age group. If the individual has serum 25(OH)D concentration less than 20 ng/mL or has persistent serum 25(OH)D less than 30 ng/mL, i.e. defined by at least 2 consecutive measurements, and with confirmed adherence, then the dose of vitamin D should be increased to step 2, i.e. 2000 IU/day for the same age group (108). Therefore, it is critical that serum 25(OH)D concentration is rechecked 3 months after the dose has been changed.

Table 2. Dietary reference intakes and recommendations from the CF Foundation for vitamin D (108, 119)

Age	**Dietary Reference Intakes (IU/day)**				**Age**	**CF Foundation Recommendations (IU/day)**		
	AI	**EAR**	**RDA**	**UL**		**Initial dose**	**Step 1**	**Step 2**
0-6 months	400	-	-	1000	0-6 months	400-500	800-1000	2000
6-12 months	400	-	-	1500	6-12 months	400-500	800-1000	2000
1-3 years	-	400	600	2500	1-10 years	800-1000	1600-3000	4000
4-8 years	-	400	600	3000				
9-13 years	-	400	600	4000				

FATTY ACID ALTERATIONS IN CYSTIC FIBROSIS

Long-chain Fatty Acid Metabolism

Linoleic acid (LA, 18:2n-6) and alpha-linolenic acid (ALA, 18:3n-3) are essential fatty acids (EFA) in humans because humans lack Δ12- or Δ15-desaturase enzymes. LA and ALA are metabolized by a series of desaturases and elongases; these pathways share the same enzymes and the first step that utilizes Δ6-desaturase is the rate-limiting step. Δ6-desaturase preferentially utilizes n-3 fatty acids as a substrate, then n-6 fatty acids, and followed by n-9 fatty acids (120). Arachidonic acid (AA, 20:4n-6) is the product of the n-6 pathway and is the dominant substrate used for eicosanoid synthesis to produce inflammatory mediators such as prostaglandins, thromboxanes, and leukotrienes. Eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are products of the n-3 pathway. Figure 1 shows an overview of long-chain fatty acid biosynthetic pathways (121).

Since these pathways compete for the same desaturases and elongases, each class of EFA can affect the metabolism of the other. An excess of n-6 EFA will reduce the metabolism of ALA and its metabolites; conversely, an excessive intake of fish oils, a major source of n-3 EFA, will decrease the metabolism of LA and its metabolites (122). In humans, the conversion of ALA to DHA was found to be less than 1% of dietary consumption and depends on other factors such as dietary n-6 FA concentrations and ratio of LA to ALA (123). In the presence of ALA and LA, Δ6-desaturase rarely uses oleic acid (18:1n-9); however, during essential fatty acid deficiency (EFAD), mead acid (20:3n-9), also known as triene, is synthesized from oleic acid, due to the lack of competitive effects of n-3 and n-6 EFAs, hence an elevated triene concentration is a marker of EFAD.

When the metabolites of n-3 and n-6 pathways, i.e. AA, EPA, and DHA, are present in the diet, they are incorporated into tissue structural lipids more efficiently than if they were synthesized from dietary LA and ALA. Membrane phospholipids contain high concentrations of polyunsaturated fatty acids (PUFA); AA is the most abundant long-chain PUFA and EPA and DHA are the most abundant n-3 FA in membrane phospholipids. The concentration of free AA is strictly regulated via phospholipases and acyltransferases. Phospholipase A_2 cleaves PUFA in position 2 of the phospholipid and the free PUFA released from the phospholipid can be metabolized for other purposes (122).

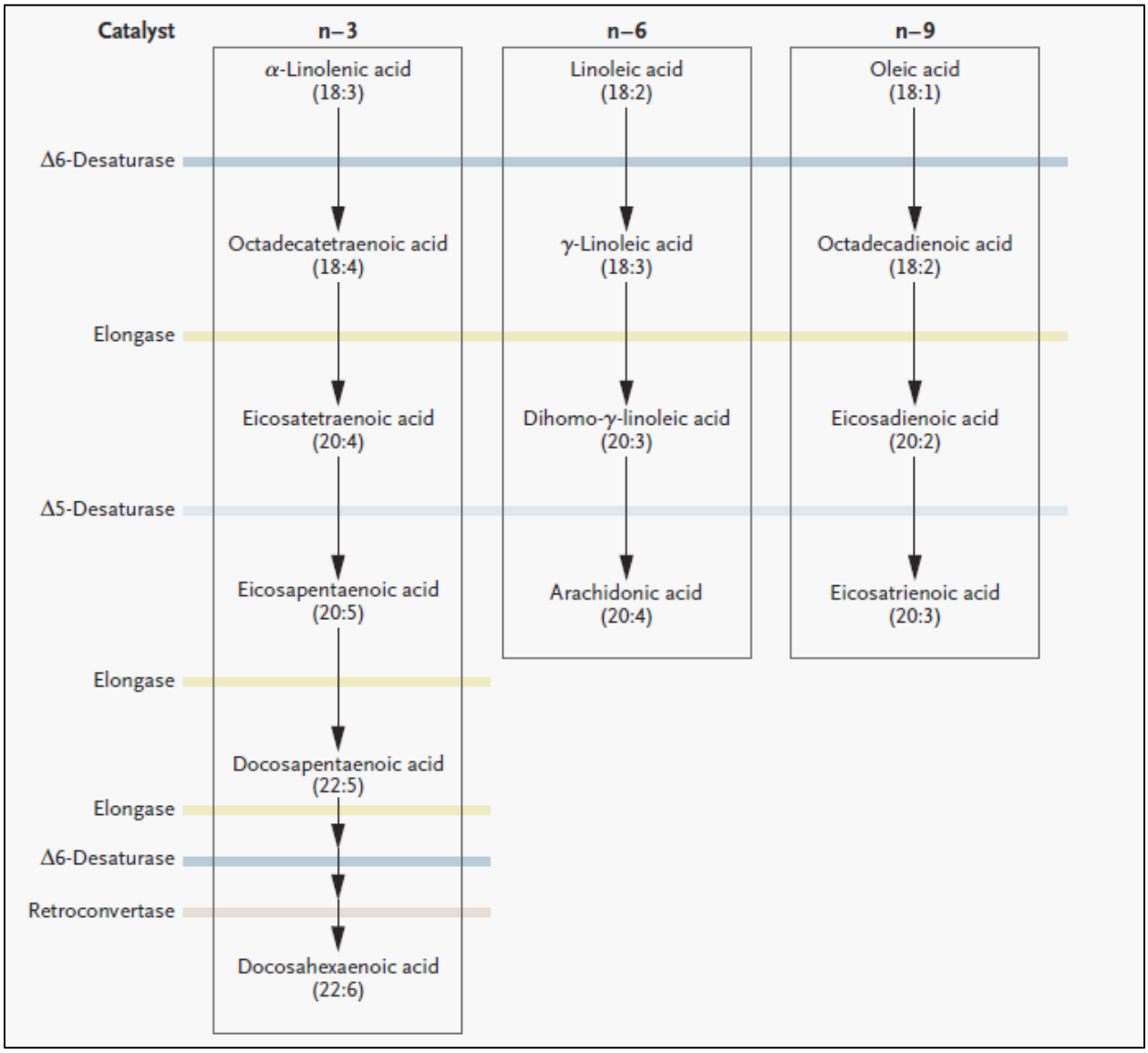

Figure 1. An overview of the biosynthetic pathways of unsaturated n-3, n-6, and n-9 fatty acids (121).

Evidence of Essential Fatty Acid Abnormalities in Cystic Fibrosis

Essential fatty acid abnormalities have been reported in the CF population (66, 121, 124-129). The most commonly reported essential fatty acid abnormality is decreased LA (66, 121, 124, 125, 127, 130, 131). An increase in mead acid production and triene:tetraene ratio, i.e. ratio of mead acid to AA, were also reported and used as biomarkers to indicate EFAD in the CF population (132, 133). Although decreased LA, increased mead acid, and increased triene:tetraene ratio are indicative of EFAD, none of the studies reported patients with clinical symptoms of EFAD such as dry, scaly skin or rash (127, 128, 133).

EFA abnormalities were previously thought to be secondary to pancreatic insufficiency but studies have found that these abnormalities were also observed in pancreatic sufficient patients (121, 134-137). Another study also found that in well-nourished patients with CF, their mead acid and palmitoleic acid (16:1n-7) concentrations are higher and LA and DHA are lower than controls, indicating that even in the absence of protein-energy malnutrition, these EFA abnormalities exist, thus EFA abnormalities in CF are more than just malabsorption or malnutrition (66, 126, 138).

One of the first studies to identify alterations in fatty acid composition in individuals with CF found increased palmitoleic acid and oleic acid and decreased palmitic acid (16:0), stearic acid (18:0), and LA in serum chylomicrons compared to controls (130). Similar findings were also reported in many studies and in different lipid fractions (66, 121, 124, 125, 127, 128, 131, 132). In addition to decreased LA, pancreatic epithelial cells derived from a CF patient showed a decrease in LA incorporation into phospholipids and increase LA incorporation into triglycerides; this study provided an important finding that EFAD in CF may be due to unbalanced intracellular utilization of LA rather than inadequate dietary EFA intake (139).

Interestingly, a study found that patients with CF had higher ALA concentrations even though their downstream metabolite, DHA, was significantly lower than their controls; this could be a result of competition with n-3 metabolites that are downstream of ALA but upstream of DHA. Moreover, the high ALA concentrations were attributed by the authors to high seafood intake in the region and dietary advice on using omega-3 rich oils such as canola oil (133).

However, findings involving AA were less consistent. Some studies found similar AA concentrations in patients with CF compared to controls (127, 132, 133), while others found that patients with CF had significantly higher AA concentrations (140-143). One study found that patients with CF had increased AA concentrations in the mucus compared to otherwise healthy controls, chronic bronchitis infected patients without CF, and *Pseudomonas*-infected patients without CF, suggesting that the increase in AA concentrations stems from a basic defect in fatty acid metabolism as a result of CFTR dysfunction, and not from lung infections (140). Yet, another study found that patients who were chronically infected with *Pseudomonas* strains had significantly higher AA concentrations than patients who were not colonized with *Pseudomonas*, indicating that AA and infections are interconnected (127). Moreover, an *in vitro* study added AA to the cell membrane of airway epithelial cells and found that it inhibited apical membrane chloride channels in both CF and normal cells. This indicated that increased AA may worsen the chloride channel defect in CF (144). One study also suggested that the decrease in LA and concomitant increase in AA observed was the result of increased flux to the n-6 pathway (145).

Association of Fatty Acids and Inflammation

In addition to airway infections, abnormal EFA metabolism could be related to inflammation in CF. Eicosanoids are lipid signaling molecules derived from 20-carbon PUFAs, mainly AA, EPA, and dihommo-gammalinolenic acid (DGLA, 20:3n-6). The rate-limiting step in eicosanoid synthesis is the release of fatty acid from phospholipids. Free AA released from the membrane phospholipids by phospholipases, especially phospholipase A_2, can be metabolized by cyclooxygenases and lipoxygenases to prostaglandins (PG), leukotrienes (LT), and hydroxyeicosatetraenoic acids (HETEs) (146). PG and LT are important effectors in immunity and inflammation.

In CF, there is an increase in phospholipase A_2 which contributes to the increase in free AA in circulation (138). PG and thromboxanes (TX) produced from free AA and interleukin (IL)-6 synthesis is stimulated by endogenous PGE_2 production, specifically linked to Cox-2 activation (147). AA oxidized by 5-lipoxygenase produced LTB_4 which is involved in neutrophil recruitment, and thus enhances the production of pro-inflammatory cytokines; 12-lipoxygenase can also act on free AA to produce 12-HETE, generating a proinflammatory cascade (122, 148). A study found that in the sputum of children with CF, tumor necrosis factor (TNF)-α is positively correlated with LTB_4 concentrations even when the subjects are clinically stable (149). Hence, the increase in AA observed in CF is a concern as it is a precursor to multiple inflammatory mediators.

Supplementation of Cystic Fibrosis Patients with Fatty Acids

According to the current CFF guidelines on management of infants with CF, there is not enough evidence to recommend for or against LA or DHA supplementation in infants with CF in

the first 2 years of life (48). There have been a few studies that have looked at EFA supplementation and its effects on children with CF (129, 150-152). One study showed that infants with CF who were on a predigested formula with higher LA content (12% kcal) had significant growth improvements compared to infants who were on a predigested formula with lower LA content (7% kcal), suggesting that infants require a higher LA content in their diet for optimal growth (151). Another study investigated effects of safflower oil (high in LA content) supplementation and observed that children with CF who were supplemented with 1g/kg/day of safflower oil for 1 year still had significantly lower LA concentrations than healthy controls and the post-supplementation plasma LA concentrations were not significantly different than pre-supplementation concentrations. Hence, this suggested that even high LA intake with safflower oil supplementation was not able to correct for EFA abnormality in CF (150).

Comparatively, a study that gave small doses of n-6 and n-3 EFA (LA, gamma-linolenic acid (GLA, 18:3n-6), EPA, and DHA) for a year in adolescents with CF found that their palmitoleic acid and AA concentrations decreased compared to baseline and DHA and LA increased compared to baseline. Together with these changes, the study also noted that post-supplementation, the concentration of TNF-α, a signaling protein involved in systemic inflammation and acute phase reaction, also decreased. One suggested theory was that GLA may have acted synergistically with n-3 FA to increase DGLA concentration, the immediate upstream metabolite of AA, and thus the decreased AA concentration was observed. This study also observed improvements in respiratory parameters such as decreased number of exacerbations and antibiotics courses, and nutritional parameters such as increased lean body mass. As such, this study showed that a low dose of a combination of n-6 and n-3 FA supplementation may be

able to normalize fatty acid composition and improve clinical parameters in patients with CF (129).

Similarly, van Biervliet et al. conducted a year-long, double-blinded, placebo-controlled trial on oral DHA supplementation in patients who were F508del homozygotes and found that DHA and EPA concentrations increased while mead acid, AA, and DGLA concentrations decreased in the treatment group post-supplementation compared to baseline (152). Even though EPA was not included in the supplement, the increase in EPA concentration observed can be attributed to the retroconversion of DHA to EPA (153). The decrease in DGLA and AA could be due to the inhibitory effects of DHA on Δ6- and Δ5-desaturases as demonstrated in cell culture models (154, 155).

Overall, it has been established that EFAD observed in the CF population is due to a basic defect in FA metabolism as a result of dysfunction of CFTR. There is currently insufficient evidence to conclude if FA supplementation, either LA or DHA, or a combination of n-3 and n-6 FA are effective in correcting the altered FA composition in the CF population. Likewise, the optimum doses of the FA supplement still need to be deliberated and these concerns regarding EFAD and FA supplementation can only be addressed by future multicenter clinical trials.

INFLAMMATION IN CYSTIC FIBROSIS

Airway infection and inflammation contribute to the progressive nature of lung disease in CF. Even in asymptomatic infants, there is evidence that airway infection and inflammation are present in infants as young as 3 months of age (22, 156, 157). Studies have found that in the bronchoalveolar lavage fluid (BAL) of infants and young children with CF, increases in inflammatory markers, such as IL-1β, IL-8, and TNF-α were observed, indicating the presence of

inflammation in the airways (22, 156, 158). Children with CF are usually initially colonized with *Staphylococcus aureus* and *Haemophilus influenzae*; as they grow older, *Pseudomonas aeruginosa* becomes the more common pathogen (2, 157).

Recurrent lung infections and neutrophil-dominated infiltration of the airways are characteristics of inflammation in CF. Inflammation in CF is a balance of pro-inflammatory and anti-inflammatory cytokines. Cytokines are small proteins secreted by cells of both innate and adaptive immune systems and can regulate diverse functions in the immune response. Cytokines are often produced in a cascade and can act synergistically or antagonistically. Cytokines also can work at extremely low concentrations (159). Pro-inflammatory cytokines are mainly produced by activated macrophages. Some examples of pro-inflammatory cytokines are IL-1β, IL-6, IL-8, and TNF-α. IL-10 is an example of an anti-inflammatory cytokine. These cytokines were selected for the purpose of this dissertation as they are the most commonly reported cytokines in the CF literature. The cytokine response in CF is usually characterized by high IL-1β, IL-6, IL-8, and TNF-α concentrations and low IL-10 concentrations (158, 160).

TNF-α is involved in systemic inflammation and is mainly activated by macrophages. IL-1β shares many similar functions as TNF-α. TNF-α and IL-1β induce the inflammatory responses of other cytokines such as IL-6 and IL-8 and augments neutrophil adhesion and neutrophil activation and migration (160, 161). IL-6 is also produced by macrophages and mediates the synthesis of and release of acute phase proteins in the liver, for example, C-reactive protein (CRP) (160, 162). IL-8 is a potent neutrophil chemoattractant in CF lungs, even in the absence of any detected pathogen in the airways (163). IL-10 is an anti-inflammatory cytokine and inhibits the synthesis of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8, as well as activation of macrophages.

Many studies have shown that TNF-α, IL-1β, and IL-8 concentrations were increased in the CF population compared to healthy controls or were increased during pulmonary exacerbations compared to when clinically stable and CF patients who were chronically infected with *Pseudomonas* (156, 158, 164). Bonfield et al. were the first to show that IL-10 concentrations in BAL were lower in CF patients than in controls (158).

In the CF population, plasma IL-6 concentrations were found to be significantly negatively correlated with FEV1 % predicted, a measure of lung function, and positively correlated with CRP concentrations, in CF patients when clinically stable and when in acute pulmonary exacerbation. The study also found that IL-6 and IL-10 concentrations in nasal lavages were significantly higher during pulmonary exacerbations than when clinically stable (165). Another study investigated the concentrations of IL-6 at the start and end of an antibiotics course and found that IL-6 concentrations were increased during the exacerbation and at the end of the course, IL-6 were significantly lower than the start of the antibiotics course. Interestingly, the IL-6 concentrations in CF patients when stable were not significantly different than those in CF patients at the start of the antibiotics. Since IL-6 mediates the synthesis of CRP, it was not unexpected that the study found that IL-6 was significantly positively correlated with CRP in subjects with or without CF (166).

Furthermore, a study that compared cytokines among CF patients with small airway disease, CF patients without small airway disease, and healthy controls found that IL-1β and IL-8 protein levels in sputum were the highest in CF patients with small airway disease, followed by CF patients, and then healthy controls. Similar findings were reported in mRNA expression of IL-1β and IL-8 in sputum which suggested that airway neutrophilic inflammation is more pronounced in CF patients with small airway disease (167).

The role of CRP as a biomarker may be more useful than just an indicator of acute inflammation in patients with CF. A study designed a severity index of exacerbations based on clinical parameters including loss of lung function after an exacerbation, recovery of lung function after an exacerbation, number of days on intravenous (IV) antibiotics, and presence of complications during the exacerbation, and explored the association between this severity index and CRP during hospital admission for pulmonary exacerbation. The study found that CRP concentrations at the start of hospital admission were not associated with the severity index but were associated with the number of exacerbations treated with IV antibiotics in the past year and colonization by *Pseudomonas aeruginosa*. However, this study did not measure CRP at more than one time point and the lack of association observed could be attributed to the use of a scoring system that was not validated (168). A prospective study that was conducted for 12 months in stable adult patients with CF found that using a cut-off concentration of 5.2 mg/L for CRP revealed that patients with baseline CRP concentrations greater than the cut-off had significantly lower FEV1 % predicted, increased frequency of pulmonary exacerbations, and worse clinical disease activity and quality of life scores than patients with baseline CRP concentrations lower than the cut-off, suggesting that CRP can be clinically useful as an indicator of clinical disease activity or risk of future exacerbations (169).

In summary, patients with CF have a basal level of inflammation when clinically stable due to CFTR defect and this level of inflammation increases during pulmonary exacerbations and bacterial colonization, predominantly as a result of neutrophils infiltration in the airways. Persistent chronic inflammation is a concern in patients with CF and there is evidence that inflammation exists even in infants with CF and contributes to the progressive nature of CF lung

disease. As such, even though cytokines are not measured routinely, it is still important to monitor inflammation clinically.

CONCLUSION AND OBJECTIVES

In conclusion, CF is characterized predominantly by gastrointestinal and pulmonary dysfunction due to the dysregulation of CFTR proteins. Maldigestion and malabsorption result in poor growth and nutrient deficiencies even after treatment with pancreatic enzymes. Caloric and vitamin supplementation are necessary to ensure optimal growth in children with CF. EFA insufficiency and deficiency are concerns but have not been a clinical focus currently. The inflammatory response in CF is also crucial and is potentially linked to EFA metabolism, which is an active area of research for both understanding the mechanisms and clinical relevance for treatment and monitoring disease progression.

The overall objective of this dissertation is to explore nutritional status, specifically vitamin D and EFA, and inflammation in a cohort of infants and young children who were diagnosed with CF via NBS in the first 2 years of life. Questions that this dissertation aims to answer include:

1. Are the current recommended doses for management of vitamin D deficiency in the CFF guidelines effective in achieving optimal vitamin D status, i.e. 25(OH)D ≥ 30ng/mL? (Chapter 2)
2. How does the serum 25(OH)D measured at various CF centers compare to our clinical measurement and how does that affect clinical interpretation of vitamin D status? (Chapter 3, presented as a short communication paper)

3. How prevalent is EFA deficiency and insufficiency in our cohort? Is there a case for monitoring EFA status routinely in practice? (Chapter 4)
4. Are there any associations in cytokines and EFA status in the first 2 years of life? (Chapter 5)
5. How does the fatty acid profile in red blood cells and plasma specimens compare since fasting plasma specimens are not possible in our cohort but is what will be measured clinically? (Appendix I)

FIRST STUDY

This dissertation utilizes a subset of the cohort enrolled in the FIRST (*Feeding Infants Right… from the STart*) study. This subcohort included subjects who were born between February 2012 to June 2016 and have reached at least 2 years of age by June 2018. The FIRST study is a multicenter, prospective, longitudinal, observational study initiated in 2012 to investigate the potential benefits and risks of exclusive breastfeeding in CF. The FIRST study originally planned to enroll 160 infants with CF identified via NBS from 2012-2016 and follow the cohort to 2 years of age in 5 excellent CF centers that were recruited based on pre-determined criteria such as the use of IRT/DNA-driven efficient diagnoses and experienced and committed pulmonologists serving as the sites' principal investigators. The excellent progress in the first 5 years of the FIRST study has enabled us to secure additional grants to expand our study to enroll 200 infants and follow them through the first 6 years of life. Phase 1 of the FIRST study is where we follow the children from diagnosis until 2 years of age and Phase 2 of the study is where we follow the children from 2 to 6 years of age.

The FIRST study was designed to test the hypothesis that prolonged exclusive breastfeeding is associated with lower risk of respiratory infections but higher risk of growth faltering in the first 2 years of life. With the addition of the follow-up from 2 to 6 years of age, we hypothesized that the pulmonary benefit of breastfeeding sustains to 6 years of age while growth faltering from exclusive breastfeeding can be lessened by a high-calorie diet at 2 to 6 years of age.

The enrollment of the FIRST study ended in 2017 and all subjects enrolled in the study have turned at least 12 months of age by December 2018. The primary outcomes for Phase 1 are weight and *Pseudomonas aeruginosa* infection at 1 and 2 years of age. Secondary outcomes include nutritional outcomes such as vitamin D status and essential fatty acid status, and pulmonary outcomes such as inflammatory markers, other respiratory pathogens, and respiratory symptoms.

We believe that the most significant aspect of the FIRST study lies in the ability to answer the question on how early malnutrition influences the onset and severity of CF lung disease. Answering this question requires longitudinal, comprehensive, and high-quality data on dietary intakes and nutritional status biomarkers beginning from the neonatal period from an unbiased birth cohort of infants with CF. To our knowledge, the FIRST study is the only study that is establishing a database with complete nutritional history in the first 6 years of life.

The aims of this dissertation are highly significant to improve clinical outcomes in CF starting in infancy. The overall objective is to evaluate the nutritional status of children with CF and its relation to inflammation and diet in the first 2 years of life. Nutritional status and pulmonary function are closely related in CF; thus, this research is essential and valuable as it critically analyzes the various aspects of nutritional status and its relation to inflammation, an

inherent characteristic of the disease. By establishing the relationships among dietary intake, nutritional status, and inflammation, we will initiate further investigations into whether nutritional intervention can reduce inflammation, thus advancing clinical care. We expect the wealth of knowledge generated from this proposed research to lead to new research direction such as EFA supplementation in infants and how it affects growth, EFA, vitamin D, and inflammatory statuses.

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CHAPTER 2

VARIABLE RESPONSE TO VITAMIN D SUPPLEMENTATION IN INFANTS AND TODDLERS WITH CYSTIC FIBROSIS

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Abstract

Background: Exocrine pancreatic insufficiency causes fat malabsorption in patients with cystic fibrosis (CF) and increases risks of vitamin D deficiency. Nationwide newborn screening for CF provided unprecedented opportunities for earlier intervention with fat-soluble vitamin supplementation beginning in early infancy to reduce vitamin D deficiency.

Objectives: To determine the prevalence of vitamin D deficiency, evaluate the effectiveness of vitamin D supplementation in optimizing serum 25-hydroxyvitamin D (25(OH)D) concentrations, and identify factors associated with suboptimal vitamin D status in children with CF in the first 2 years of life.

Design: Data from an ongoing multicenter prospective longitudinal observational study at 5 CF centers in the US were analyzed. Serum 25(OH)D was quantified at 3 time points in the first 2 years of life and vitamin D supplementation and intake were assessed at routine CF visits and compared to that reported in NHANES 2009-2012.

Results: Serum 25(OH)D concentrations and vitamin D supplement intake increased significantly with age. The overall prevalence of suboptimal vitamin D status is 32% at baseline, 25% at 1 year of age, and 21% at 2 years of age. Approximately 12% of subjects did not have an increase in serum 25(OH)D concentration following an increase in vitamin D supplement intake in the first 2 years of life and continued to have suboptimal vitamin D status. Our study reported a lower proportion of children with serum 25(OH)D <30 ng/mL (20%) compared to that of NHANES (35%). Meconium ileus is a significant predictor for vitamin D responder status.

Conclusion: Approximately half of the subjects achieved optimal 25(OH)D concentrations in early infancy and maintained it through 2 years of age, a quarter responded later at 12 or 24

months of age, and 20% did not achieve optimal vitamin D status. Our study recommends earlier monitoring of bone health for children who have persistent suboptimal vitamin D status.

Keywords: cystic fibrosis, vitamin D, infants, supplementation, meconium ileus

INTRODUCTION

Cystic fibrosis (CF), one of the most common autosomal recessive diseases in the Caucasian population, is a progressive disease resulting from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Exocrine pancreatic insufficiency due to CFTR dysfunction leads to malabsorption, particularly of fat, hence fat-soluble vitamin status in individuals with CF has been a longstanding concern (1). Routine measurement of serum 25-hydroxyvitamin D (25(OH)D) to assess vitamin D status in all individuals with CF is recommended in the clinical practice guidelines by the Cystic Fibrosis Foundation (CFF) (2-4). According to current CFF guidelines, optimal vitamin D status is defined as serum 25(OH)D concentration above 30 ng/mL which is equivalent to 75 nmol/L (4). The recommended initial dose for infants with CF from birth to 12 months of age is 400-500 IU/day and is increased to 800-1000 IU/day for children older than 12 months of age.

Vitamin D deficiency is associated with poor skeletal health, muscle weakness, autoimmune diseases, and other diseases associated with non-skeletal metabolic pathways of vitamin D (5-7). Low concentrations of serum 25(OH)D were found to be directly related to low bone mineral density (8); particularly in individuals with CF, low bone mineral density was found to be common at a young age (9, 10). Many studies have examined vitamin D status in older children and young adults with CF (1, 7, 11, 12). The reported prevalence of vitamin D insufficiency (serum 25(OH)D <30 ng/mL) ranged widely from 23% to 90% (11, 12), most likely due to differences in the study population's quantity of vitamin D intake and season of 25(OH)D measurement. However, few studies have focused on vitamin D status in infants with CF primarily because this population was not available to study extensively until nationwide newborn screening (NBS) for CF was implemented in 2010 (13).

An older 13-year prospective study conducted in Colorado in infants diagnosed with CF via NBS found vitamin D deficiency (serum 25(OH)D <14 ng/mL) in 22.5% of infants at 2 months, 4.2% at 6 months, 2.4% at 12 months, and 5.1% at 24 months (14). A more recent prospective study conducted in the US observed that 51% of infants diagnosed via NBS had serum 25(OH)D <30 ng/mL at around 1 year of age (15). However, this study only reported a one-time snapshot of vitamin D status and did not further investigate factors contributing to suboptimal vitamin D status.

The present study aims to examine the vitamin D status of a cohort of infants and toddlers with CF diagnosed through NBS and enrolled in a multicenter, prospective, longitudinal study initiated in 2012. Specifically, we determined the prevalence of suboptimal vitamin D status, compared it to that of the apparently healthy population using data from the National Health and Nutrition Examination Survey (NHANES), examined if vitamin D supplementation according to CFF's recommended regimen is effective in achieving vitamin D sufficiency, and explored other factors that may contribute to suboptimal vitamin D status in infants and toddlers with CF.

SUBJECTS AND METHODS

Study design and population

The study population consisted of infants born between February 2012 and June 2016, diagnosed through newborn screening, and enrolled in FIRST (*Feeding Infants Right… from the STart*), a multicenter, prospective, observational study conducted at 5 CF centers (Boston, MA, Indianapolis, IN, Salt Lake City, UT, Madison and Milwaukee, WI) to identify optimal feeding for infants with CF. The inclusion criteria for recruitment include the following: diagnosis of CF either by the presence of 2 disease-causing CFTR mutations and/or by quantitative sweat chloride test (≥60 mmol/L), first CF center visit before 10 weeks of age, and routine follow-ups at one of the 5 CF centers. The study was approved by the Institutional Review Boards at the University of Wisconsin-Madison and all participating institutions. Parents of all subjects provided written informed consent.

A total of 178 infants born in 2012-2017 were enrolled in the FIRST study at age 1.8 ± 1.0 months (mean ± SD). Among them, 141 subjects were at least 24 months of age by 06/30/2018. Five infants withdrew before 3 months of age, 9 had a low birth weight (<2500 g), and another 5 had no blood drawn during routine visits in the first 2 years of life; the final analyses were done on 122 subjects **(Figure 1)**.

Classification of gastrointestinal phenotype and pancreatic status

The most severe gastrointestinal phenotype in infants with CF is the neonatal presentation of meconium ileus (MI), which occurs in approximately 20% of infants with CF (16, 17). In the FIRST study, fecal specimens were collected at enrollment (approximately 2 months), 4, 6, 8, 12, and 24 months of age. All infants born with MI were also pancreatic insufficient (PI). Infants

were classified as pancreatic sufficient (PS) if their fecal elastase-1 concentrations were consistently >200 μg/g in their first 2 years of life. Therefore, we classified our study population into 3 gastrointestinal phenotypes – MI, PI, and PS.

Data collection

The FIRST study was designed such that study visits coincided with routine clinical care (monthly after diagnosis until 6 months of age, bi-monthly from 6 to 12 months of age, and every 3 months thereafter (3)). At each clinic visit, nutritional and pulmonary interval history forms were completed by trained clinic/study staff. The nutritional form included questions such as type of feeding, feeding preparation, and use of pancreatic enzyme replacement therapy and fat-soluble vitamins and the pulmonary form recorded any acute respiratory infections and hospitalizations during the past interval as well as inhaled antibiotics use for *Pseudomonas aeruginosa* eradication. Clinical data were verified with electronic medical records obtained and discrepancies were clarified by further contact with clinic staff and families. Data were entered to Online Collaborative Research Environment-Clinical Research Management (OnCore), a HIPAA-compliant online data management tool.

In addition to completion of the nutrition interval history form, parents of study subjects were asked to complete a 3-day food record prior to the clinic visits monthly before 6 months of age, bi-monthly between 6 and 12 months of age, and once at 18 months and 24 months of age. Three-day food records were analyzed by a research dietitian experienced in analyzing food records in patients with CF (16, 18) using Food Processor Nutrition Analysis Software (ESHA Research, Salem, OR).

Assessment of vitamin D intake

To ensure representation of intake at each time point and address variation in diet as a child grows, 3-day food records were included for analyses if the dates fell within a month of the 4-month blood draw, 2 months of the 12-month blood draw, and 3 months of the 24-month blood draw. When a 3-day food record was not available, the dietary intakes of vitamin D and calcium were estimated from the consumption of milk and commercial products (e.g. PediaSure®) recorded in the nutrition interval history form collected during the clinic visit when blood was obtained to measure 25(OH)D.

Measurement of 25(OH)D concentrations

Blood specimens were obtained at approximately 4 months (4M), 12 months (12M), and 18 to 24 months (24M) of age. Serum from all study specimens was sent to the Clinical Laboratory Services of the University of Wisconsin Hospitals and Clinics within 2 days of receipt for quantification of 25(OH)D concentrations using high-performance liquid chromatography (HPLC) with UV detection (19). Serum 25(OH)D ≥30 ng/mL is defined as vitamin D sufficient, 20 to 29 ng/mL is defined as insufficient, and <20 ng/mL is defined as deficient.

Comparison of 25(OH)D concentrations between study and local labs

Three out of 5 CF centers in the FIRST study also measured serum 25(OH)D at their respective clinical laboratories. Serum 25(OH)D from local and study clinical laboratories, if available for the same specimen, were compared to assess agreement among clinical laboratories. According to the Vitamin D Standardization Program, a clinical laboratory 25(OH)D assay

should have a CV ≤10% to be defined as traceable to the standardized reference measurement procedure (20, 21). Therefore, for our purpose, if the local serum 25(OH)D value was within 10% of the paired study clinical serum 25(OH)D, then the paired observations were considered to be the same. Paired local and study clinical serum 25(OH)D values were also analyzed based on their corresponding classification of vitamin D status, i.e. deficiency, insufficiency, and sufficiency.

Classification of responder status

We have classified our study population (n=122) into various responder status categories based on serum 25(OH)D findings (Figure 1). Only specimens that were collected during routine follow-up visits, i.e. not during hospitalizations or visits for acute illness were analyzed. Responder status categories were defined based on baseline (~ 4M) and at least 1 follow-up serum 25(OH)D concentrations measured in the first 2 years of life. There were 17 subjects who did not have serum 25(OH)D measured at 4M or did not have a follow-up serum 25(OH)D measured. Among the remaining 105 subjects, we have classified them into 5 responder status categories.

Early responders (ER, n=56) were subjects who had serum 25(OH)D ≥30 ng/mL at 4M, 12M, and 24M. Late responders (LR, n=28) were subjects who had serum 25(OH)D <30 ng/mL at 4M or 12M and responded at 12M or 24M. Transient responders (TR, n=9) were subjects who had serum 25(OH)D ≥30 ng/mL at 4M and decreased to <30 ng/mL at 12M and/or 24M. Non-responders (NR, n=12) were those who either had serum 25(OH)D <30 ng/mL at all 3 time points or had serum 25(OH)D <30 ng/mL at 4M, increased to ≥30 ng/mL at 12M, and decreased to <30 ng/mL again at 24M.

Vitamin D status of children in the NHANES population

Serum 25(OH)D concentrations, vitamin D intake from two 24-hour recalls, and demographics of survey participants were obtained from NHANES for the years 2009-2010 (https://wwwn.cdc.gov/Nchs/Nhanes/2009-2010/VID_F.XPT) and 2011-2012 (https://wwwn.cdc.gov/Nchs/Nhanes/2011-2012/VID_G.XPT). As described in the NHANES survey operations manual, serum 25(OH)D was measured via ultra-HPLC-tandem mass spectrometry (UHPLC-MS/MS) (22).

Statistical analysis

Statistical analyses were performed using SAS (v9.4, SAS Institute Inc., Cary, NC) and graphs were plotted using SigmaPlot (v13.0, Systat Software, San Jose, CA) and GraphPad Prism (v8 GraphPad Software, La Jolla, CA). Data were presented as mean ± SD unless otherwise stated; p values <0.05 were considered as significant. Data were checked for normality using Shapiro-Wilk test; if data were not normally distributed, a non-parametric test was used. Chi-square test was used to determine the association between 2 categorical variables and Fisher's exact test was used in place of chi-square when the cell sizes were expected to be less than 5. T-test and Mann-Whitney U test, where applicable, were used to determine whether the means of 2 groups differ significantly from each other. ANOVA and Kruskal-Wallis (where applicable) were used to compare differences among group means and medians, respectively. Agreement between local and study values of 25(OH)D concentrations was assessed by using the kappa statistic. A kappa of <0.4 was considered to be in poor agreement according to Landis and Koch (23). Linear regression models were used to explore the association between serum 25(OH)D concentrations and age, as well as vitamin D supplement intake and age. Logistic

regression was used to assess the association among responder groups, using sex and phenotype categories as covariates.

RESULTS

Characteristics of study population

Of the 122 subjects included in the analyses, most were non-Hispanic white (92.6%), diagnosed via NBS (89.3%), and were PI (68.0%). **Table 1** summarized the characteristics of the study population including gender, racial and ethnicity categories, GI phenotype, F508del genotype, age at first CF center visit, number of upper respiratory tract infections requiring antibiotics, hospitalizations, and *Pseudomonas aeruginosa* acquired in the first 2 years of life by vitamin D responder status.

Comparison of local clinical measurements to study clinical measurements

There were 103 pairs of local and study blood specimens available for pairwise comparison of serum 25(OH)D concentrations. Most local serum 25(OH)D values were either within 10% (74.8%) or higher (24.3%) than study clinical serum 25(OH)D values. Given a 10% margin of each other, 88 pairs (85.4%) had the same vitamin D status classification, i.e. ≥30 ng/mL or <30 ng/mL and overlapped with each other; 10 pairs had the same vitamin D status classification but do not overlap, whereas 5 pairs do not have the same vitamin D status classification. This indicated that there was a less than 5% chance of misclassifying vitamin D status if we used the local serum 25(OH)D measurement. Furthermore, the overall kappa was calculated to be 0.564 and ranged from 0.429 to 0.731 when analyzed by CF center. Therefore, if a study serum 25(OH)D measurement was not available for analysis, the local clinical measurement was used. Of the 316 observations used in the analyses, 35 (11.1%) were local clinical measurements.

Serum 25(OH)D concentrations and vitamin D supplement intake in the first 2 years of life

Serum 25(OH)D concentrations and vitamin D supplement intake in the first 2 years of life increased with age, p=0.014 and p<0.001, respectively (**Figure 2A and 2B**). Overall, 12% of those who were PS, 27% of those who were PI, and 33% of those with MI had suboptimal vitamin D status, i.e. <30 ng/mL; subjects with MI had a significantly higher prevalence of suboptimal vitamin D status than those who were PI or PS, p=0.047 (**Figure 3**). Those who were PS were taking a vitamin D supplement at approximately half (46.5% of all observations) of the time. Excluding subjects who were PS, at baseline (n=93), the median intake of vitamin D supplement was 500 IU/day; this was increased to 800 IU/day at 12M (n=84) and 1350 IU/day at 24M (n=96).

Responder status according to serum 25(OH)D concentrations in the first 2 years of life

Among subjects who had a blood drawn at approximately 4M and at least 1 follow-up blood draw (12M and/or 24M, n=105), 53.3% were early responders (ER), 26.7% were late responders (LR), 8.5% were transient responders (TR), and 11.4% were non-responders (NR) as shown in Figure 1.

In LR, excluding those who were PS, for the 12 subjects whose serum 25(OH)D <30 ng/mL at 4M and increased to ≥30 ng/mL at 12M, 25% (n=3, subjects 2, 9, 10) did not have an increase in vitamin D supplement from 4M to 12M despite having 25(OH)D <30 ng/mL at 4M, as shown in **Figures 4A and 4B**. However, 2 (subjects 2 and 10) out of these 3 subjects started out at ≥900 IU/day of vitamin D, only 1 subject (subject 9) remained at 400 IU/day from 4M to 12M. There were 7 subjects who had 25(OH)D <30ng/mL in both 4M and 12M (subjects 13 to 19); 3 subjects (subjects 13, 16, 19) did not increase vitamin D supplement intake from 4M to

12M and only 1 subject (subject 15) did not increase vitamin D supplement intake from 12M to 24M; however, this subject's 12M's vitamin D intake was already at 2400 IU/day. The remaining 7 subjects in the LR group (subjects 14 to 26) had 25(OH)D ≥30 ng/mL at 4M and 25(OH)D <30 ng/mL at 12M; 100% increased their vitamin D supplement intake from 12M to 24M and achieved 25(OH)D ≥30 ng/mL at 24M (**Figures 4A and 4B**).

In TR, all subjects had 25(OH)D ≥30 ng/mL at 4M and declined to <30 ng/mL at 12M and/or 24M (**Figures 5A and 5B**). There were 6 subjects (subjects 1, 2, 4, 5, 6, 7) who had serum 25(OH)D <30 ng/mL at 12M and 24M; only 2 subjects (subjects 3 and 8) had 25(OH)D ≥30 ng/mL at 12M and declined to <30 ng/mL at 24M. Of the 6 subjects with 25(OH)D <30 ng/mL at 12M and 24M, 3 (subjects 2, 4, 6) did not increase vitamin D supplement intake from 4M to 12M and all 6 increased vitamin D supplement intake from 12M to 24M. The remaining 2 subjects (subjects 3 and 8) had an increase in vitamin D supplement intake from 4M to 12M and neither increase vitamin D supplement intake from 12M to 24M.

For the subjects who had low 25(OH)D concentrations consistently in the first 2 years of life, i.e. NR, 91.7% (n=11, all but subject 9) increased vitamin D intake from 4M to 24M, however, their 24M's 25(OH)D were still <30 ng/mL; only 1 subject (subject 9) consumed vitamin D at 400 IU/day at all 3 time points (**Figures 5C and 5D**).

Overall, at baseline, approximately 15% of those who had serum 25(OH)D ≥30 ng/mL became TR eventually despite increases in vitamin D supplement. Another 12% of subjects did not respond to increases in vitamin D supplement in the first 2 years of life. There were approximately 24% of subjects who had an increase in serum 25(OH)D concentrations in response to an increase in vitamin D supplement intake at 12M and/or 24M.

Factors associated with vitamin D responder status excluding subjects who were PS

Comparing non-responders (TR and NR) to responders (ER and LR), the number of upper respiratory tract infections requiring antibiotics, hospitalizations, and *Pseudomonas aeruginosa* acquired in the first 2 years of life were not significantly associated with vitamin D responder status (**Table 2**). Vitamin D supplement intake as a dichotomous variable, i.e. at least once ≥ recommended or always ≥ recommended per guidelines, was also not significantly associated with vitamin D responder status.

For the comparison within the responder groups, i.e. LR vs. ER, the number of upper respiratory tract infections requiring antibiotics, hospitalizations, and *Pseudomonas aeruginosa* acquired in the first 2 years of life were not significantly associated with vitamin D responder status (Table 2). However, GI phenotype was significant for all models; having a history of MI is significantly less likely than those who were PI to be in the ER group, $p<0.03$. In addition, for all models, those whose vitamin D supplement intakes that were not always at the recommended level were significantly more likely (OR range from 9.5 to 13.5) than those whose vitamin D supplement intakes were at or above the recommended level at all times in the first 2 years of life to be in the ER group, $p<0.05$. Most (60%) of those who did not always consume vitamin D supplement at the recommended level were from ER. Additionally, 29.5% of ER had vitamin D supplement intake less than 800 IU/day, which is the recommended intake level.

Vitamin D status of 2009-2012 NHANES population

In the combined 2009-2012 NHANES population, there were 85 non-Hispanic White with serum 25(OH)D measured and dietary intake recorded at 2 years of age. Of these 85 children, 35.3% had serum 25(OH) <30ng/mL, including 1 (1.2%) who had serum 25(OH)D <20ng/mL **(Table 3)**. Dietary vitamin D intake is also summarized in Table 3; 45.9% of the

children consumed dietary vitamin D below the estimated average requirement (EAR) of 400 IU/day. None of the children in the NHANES population consumed vitamin D more than the tolerable upper intake levels (UL) of 2500 IU/day.

When compared to the NHANES population, subjects in the FIRST study had significantly higher mean serum 25(OH)D concentrations, $p<0.001$, and lower proportion of children with serum 25(OH)D <30 ng/mL, $p=0.016$ (Table 3). Subjects enrolled in the FIRST study also had a higher proportion of children consuming vitamin D from diet below the EAR, $p=0.007$ and lower proportion of children consuming vitamin D from diet between the recommended dietary allowance (RDA) and UL, $p=0.033$.

DISCUSSION

In our study, we found that approximately 26% of infants and toddlers in their first 2 years of life had suboptimal vitamin D status. The prevalence of suboptimal vitamin D status in the FIRST study is lower than that reported in a similar (BONUS) study (15). In BONUS, 51% of 205 infants had suboptimal vitamin D status at approximately 12 months of age, including 14% who were vitamin D deficient (serum 25(OH)D <20 ng/mL). Our study revealed that for the same age group, 25% of infants had suboptimal vitamin D status, of which only 2% were vitamin D deficient. One explanation for this discrepancy is that the serum 25(OH)D concentrations reported in the BONUS study were measured locally at each CF center (total of 28 CF centers) whereas for our study, most (88.9%) of the serum 25(OH)D were measured at the Clinical Laboratory Services at the University of Wisconsin Hospitals and Clinics. Variation in serum 25(OH)D assays has been a concern in diagnosing hypovitaminosis D (24, 25), therefore, we compared the local serum 25(OH)D and study serum 25(OH)D measurements in our study which showed that given a 10% margin of each other, 95.1% of the local-study pairs had the same classification of vitamin D status and thus, there is a less than 5% error of misclassification of vitamin D status in our study if we had used a local serum 25(OH) measurement.

Additionally, a study that looked at vitamin D status in infants with CF showed that 25(OH)D concentrations in infants who were diagnosed via NBS, within the first 3 weeks of diagnosis, were not significantly different between the PI and PS group according to retrospective data (26). However, our study has shown that approximately 13% of PS infants had suboptimal vitamin D status whereas 38% of PI infants had suboptimal vitamin D status at baseline, implying that pancreatic status does have a role to play in vitamin D status.

Our paper is one of few papers to explore vitamin D status in similar age groups reported in NHANES. Even though the proportion of children in NHANES consuming vitamin D at or more than the RDA was significantly higher compared to children in FIRST, our study found that the prevalence of suboptimal vitamin D status is lower than that of the national average (19.6% in FIRST vs. 35.3% in NHANES) at 2 years of age (Table 3). This indicated that with vitamin D supplementation as recommended in the current vitamin D management regimen for infants and toddlers with CF, they fare better in terms of vitamin D status compared to the otherwise healthy average children. It also showed that vitamin D supplementation is effective for most of the children with CF to optimize their vitamin D status to that of the otherwise healthy population.

To our knowledge, this is the first paper to look at individual responses to vitamin D supplementation on vitamin D status in the first 2 years of life for children with CF. Our study found that even with early and sustained high daily doses of vitamin D, only about 24% responded to the increase in vitamin D supplementation, whereas another 12% continued to have suboptimal vitamin D status. At baseline, about 14% of subjects did not have their serum 25(OH)D measured and about 15% of those who were vitamin D sufficient at 4M eventually had suboptimal vitamin D status by 2 years of age, thereby emphasizing the need to monitor vitamin D status early on. There were few subjects who had serum 25(OH)D <30 ng/mL and did not have an increase in vitamin D supplementation which could be due to clinicians' discretion or the choice of caregivers.

Moreover, we have identified that having a history of MI decreased the odds of being in the ER group. MI is the most severe manifestation of CF at birth and was previously found to be associated with malnutrition and malabsorption as a result of surgical treatment of MI (16),

hence, it is not surprising that MI is a significant predictor of vitamin D status in the first 2 years of life for children with CF. Additionally, we also found that those who were not consuming vitamin D supplements at the recommended intake for all 3 time points in the first 2 years of life were 9.5 to 13.5 times more likely to be in the ER group. One reason was that subjects who have optimal vitamin D status, i.e. serum 25(OH)D ≥30 ng/mL may not increase their vitamin D supplement intake if their serum 25(OH)D continued to be at or above 30 ng/mL, i.e. ER. Therefore, even at 2 years of age, almost 30% of ER were not consuming vitamin D supplements at the minimum recommended intake of 800 IU/day.

A history of hospitalizations and *Pseudomonas aeruginosa* acquisition did not appear to affect the vitamin D status responder group classification. This could be because subjects who were hospitalized at least once would have been monitored for overall nutritional status and have follow-up visits allowing for more opportunities for intervention if they had suboptimal vitamin D status. In addition, most subjects (78%) had not acquired *Pseudomonas aeruginosa* by 2 years of age.

Apart from bone health, suboptimal vitamin D status has been associated with pulmonary exacerbations (27) and forced expiratory volume in 1 second (FEV_1) % predicted (28). Our study population was too young for pulmonary function tests to be carried out during routine visits and we were not able to assess the association between vitamin D status and FEV_1 % predicted. One limitation of our study was that it was designed to be a comprehensive observational study, so further research will be needed to explore the factors contributing to the variable responses in vitamin D supplementation. For example, a study identified single nucleotide polymorphisms (SNPs) in a vitamin D supplementation randomized controlled trial in older adults (29). The study confirmed associations between 25(OH)D concentrations and 12 SNPs found in CYP2R1

(25-hydroxylase) and GC (vitamin D-binding protein) genes. Both genes are involved in the vitamin D metabolic pathway and contribute to 25(OH)D in circulation. This may explain the variable responses observed in our study, although it is not known how these genes interact with the pathology of CF. Additionally, our study limited our analyses to subjects who had baseline serum 25(OH)D concentration measured to maximize our data points and sample size over the 2 years; a longer-term longitudinal study will be able to evaluate vitamin D status more comprehensively.

For the 12% who had persistent suboptimal vitamin D status, the supplement doses ranged from 400 to 2800 IU/day. We suggest giving the maximum dose of 4000 IU/day for children 1 year and above in order to increase the chances of responding to higher doses. It is recognized that vitamin D toxicity (serum 25(OH)D >100 ng/mL) can lead to hypercalcemia, however, vitamin D toxicity is an uncommon occurrence even in the CF population where vitamin D intake is high (30, 31). The highest 25(OH)D concentration recorded in our study was 75 ng/mL for a subject with PS with an intake of 1000 IU/day and the highest vitamin D intake recorded was 3200 IU/day and the corresponding 25(OH)D concentration was 44 ng/mL. As such, there is minimal risk of toxicity in a population where malabsorption is a common occurrence.

The primary concern with vitamin D deficiency is bone health. According to current guidelines for bone disease in CF, it is recommended that children above 8 years old with <90% of ideal body weight be screened using dual energy x-ray absorptiometry (DXA) (32). In view of the variable responses to vitamin D supplementation, our study suggests monitoring bone mineral density before 8 years of age during this crucial bone accrual period. Since DXA may be

unreliable in younger children, a quantitative computed tomography can be an alternative as suggested in an updated CF-related bone disease review (33).

Our study showed that the current supplementation regimen is effective in most children with CF and responses to supplementation is variable. More research is needed to investigate other factors such as genetic variation in order to develop a more personalized approach to vitamin D supplementation in CF.

CONCLUSION

Our study showed that in the first 2 years of life, about half of infants and toddlers with CF have optimal vitamin D status with vitamin D supplementation; a quarter achieved optimal vitamin D status by 2 years of age, following an increase in vitamin D supplement, despite having suboptimal vitamin D status at baseline. Approximately 10% never responded to the increase in vitamin D supplementation and had suboptimal vitamin D status throughout the first 2 years of life. Having a history of MI is a significant predictor for vitamin D status. Genetic variation in genes involved in the vitamin D metabolic pathways may also contribute to vitamin D status. Therefore, we recommend monitoring bone health earlier for children with MI or who have persistent suboptimal vitamin D status.

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Table 1. Characteristics of study population (n=122), born between February 2012 and June 2016[1]

		By vitamin D responder status[2]				
	All (n=122)	**Early Responders (ER) (n=56)**	**Late Responders (LR) (n=28)**	**Transient Responders (TR) (n=9)**	**Non-Responders (NR) (n=12)**	**Not classified (n=17)**
Male	66	30 (45.5)	17 (25.8)	5 (7.6)	4 (6.1)	10 (15.2)
Racial/ethnicity categories						
Non-Hispanic White	113	55 (48.7)	27 (23.9)	7 (6.2)	11 (9.7)	13 (11.5)
Other[3]	9	1 (11.1)	1 (11.1)	2 (22.2)	1 (11.1)	4 (44.4)
Diagnosis						
Newborn screening	109	47 (43.1)	25 (22.9)	9 (8.3)	12 (11.0)	16 (14.7)
Prenatal screening	13	9 (69.2)	3 (23.1)	0 (0)	0 (0)	1 (7.7)
GI phenotype						
MI	22	4 (18.2)	8 (36.4)	6 (27.2)	1 (4.5)	3 (13.6)
PI	83	41 (49.4)	18 (21.7)	2 (2.4)	11 (13.3)	11 (13.3)
PS	17	11 (64.7)	2 (11.8)	1 (5.9)	0 (0)	3 (17.6)
Genotype						
F508del/F508del	59	26 (44.1)	16 (27.1)	6 (10.2)	7 (11.9)	4 (6.8)
F508del/other	53	24 (45.3)	9 (17.0)	3 (5.7)	5 (9.4)	12 (22.6)
Other/other	10	6 (60.0)	3 (30.0)	0 (0)	0 (0)	1 (10.0)
Age at first CF center visit (month)[4]	0.70[5] ± 0.62 0.52[6]	0.60 ± 0.43 0.46	0.71[7] ± 0.47 0.49[7]	1.25 ± 0.55 1.15	0.57 ± 0.35 0.56	0.74 ± 0.42 0.51
Vitamin D supplement intake[8]						
At least once ≥ recommended	20	12 (60.0)	1 (5.0)	1 (5.0)	1 (5.0)	5 (25.0)
Always ≥ recommended	85	33 (38.8)	25 (29.4)	7 (8.2)	11 (12.9)	9 (10.6)
Upper respiratory tract infections[9]						
0-1	34	15 (44.1)	7 (20.6)	6 (17.6)	2 (5.9)	4 (11.8)
≥2	88	41 (46.6)	21 (23.9)	3 (3.4)	10 (11.4)	13 (14.8)
Hospitalizations[10]						
None	67	31 (46.3)	16 (23.9)	5 (7.5)	9 (13.4)	6 (9.0)
≥1	55	25 (45.5)	12 (21.8)	4 (7.3)	3 (5.5)	11 (20.0)
Pseudomonas aeruginosa acquisition[11]						
None	99	45 (45.5)	22 (22.2)	8 (8.1)	9 (11.0)	15 (15.2)
≥1	23	11 (47.8)	6 (26.1)	1 (4.3)	3 (13.0)	2 (8.7)

[1]Values are *n* (%) for the corresponding row unless otherwise indicated
[2]See Figure 1 for definition and classification of vitamin D responder status
[3]Other includes American Indian or Alaska Native, Asian, Black or African American, Hispanic or Latino, or more than one race according to National Institutes of Health's definitions for racial and ethnic categories
[4]Excluded subjects with MI
[5]Values are mean ± SD
[6]Values are median
[7]n=19, excluded a subject who had his first visit at 4.98 months of age
[8]Per guidelines, the minimum recommended intake is 400 IU/day at 4M & 12M, and 800 IU/day at 24M. The numbers exclude those who were PS
[9]Number of upper respiratory tract infections requiring antibiotics in the first 2 years of life
[10]Number of hospitalizations in the first 2 years of life, excluding hospitalization for MI
[11]Number of *Pseudomonas aeruginosa* acquired in the first 2 years of life

Table 2. Factors associated with vitamin D responder status[1]

		Non-responders vs. Responders		Within responders	
		(TR + NR) vs. (ER + LR)		LR vs. ER	
Models	**Factors included in the multiple logistic regression models[2]**	**OR**	**p**	**OR**	**p**
1	Upper respiratory tract infections[3]				
	≥2 vs. 0-1	2.188	0.174	2.662	0.163
	GI phenotype				
	MI vs. PI	0.397	0.116	0.154	0.015
	Vitamin D supplement intake[4]				
	At least once ≥ recommended vs. Always ≥ recommended	1.795	0.481	12.387	0.027
2	Hospitalizations[5]				
	Ever vs. none	1.998	0.214	1.175	0.767
	GI phenotype				
	MI vs. PI	0.356	0.083	0.193	0.026
	Vitamin D supplement intake[4]				
	At least once ≥ recommended vs. Always ≥ recommended	1.590	0.579	9.473	0.045
3	*Pseudomonas aeruginosa* acquisition[6]				
	Ever vs. none	1.367	0.637	1.569	0.488
	GI phenotype				
	MI vs. PI	0.399	0.115	0.183	0.023
	Vitamin D supplement intake[4]				
	At least once ≥ recommended vs. Always ≥ recommended	1.779	0.489	10.685	0.037
4	Upper respiratory tract infections[3]				
	≥2 vs. 0-1	1.994	0.243	2.593	0.192
	Hospitalizations[5]				
	Ever vs. none	1.840	0.285	0.922	0.889
	Pseudomonas aeruginosa acquisition[6]				
	Ever vs. none	1.104	0.884	1.423	0.602
	GI phenotype				
	MI vs. PI	0.341	0.079	0.148	0.014
	Vitamin D supplement intake[4]				
	At least once ≥ recommended vs. Always ≥ recommended	1.674	0.543	13.580	0.026

[1]Reference category of vitamin D responder status and variables listed was the latter (e.g. 0-1 is the reference category for infections)
[2]Adjusted for sex and excluded subjects who were PS
[3]Number of upper respiratory tract infections requiring antibiotics in the first 2 years of life
[4]For vitamin D supplement intake, the minimum recommended intake is 400 IU/day at 4M & 12M, and 800 IU/day at 24M
[5]Number of hospitalizations in the first 2 years of life, excluding hospitalization for MI
[6]Number of *Pseudomonas aeruginosa* acquired in the first 2 years of life
[7]Illness severity refer to number of upper respiratory tract infections requiring antibiotics, hospitalizations, and *Pseudomonas aeruginosa* acquisition combined. None refers to subjects who never had more than 1 infection requiring antibiotics, never hospitalized, and no *Pseudomonas aeruginosa* cultured in the first 2 years of life; severe refers to subjects who had more than 1 infection requiring antibiotics, hospitalized, and *Pseudomonas aeruginosa* acquired in the first 2 years of life. Intermediate refers to all other subjects between the none and severe classification.

Table 3. Comparison of serum 25(OH)D concentrations and vitamin D intake from 2009-2012 non-Hispanic White NHANES population (n=85) to the FIRST cohort (n=102)

	24-month		
	NHANES (n=85)	**FIRST (n=102)[1]**	**p**
Serum 25(OH)D (ng/mL)[2]	33.1 ± 6.6	38.0 ± 9.9	<0.001
<30 ng/mL (%)	35.3	19.6	0.016
<20 ng/mL (%)	1.2	1.0	1.000
Intake from diet[3]			
<EAR (%)	45.9	65.7	0.0067
EAR – <RDA (%)	30.6	22.6	0.213
RDA – ≤UL (%)	23.5	11.8	0.033
>UL (%)	0	0	-

[1]There were 3 subjects who did not have dietary intake data
[2]Values are mean ± SD
[3]For 1-3 years old, estimated average requirement (EAR) is 400 IU/day, recommended daily allowance (RDA) is 600 IU/day and upper level intake (UL) is 2500 IU/day.

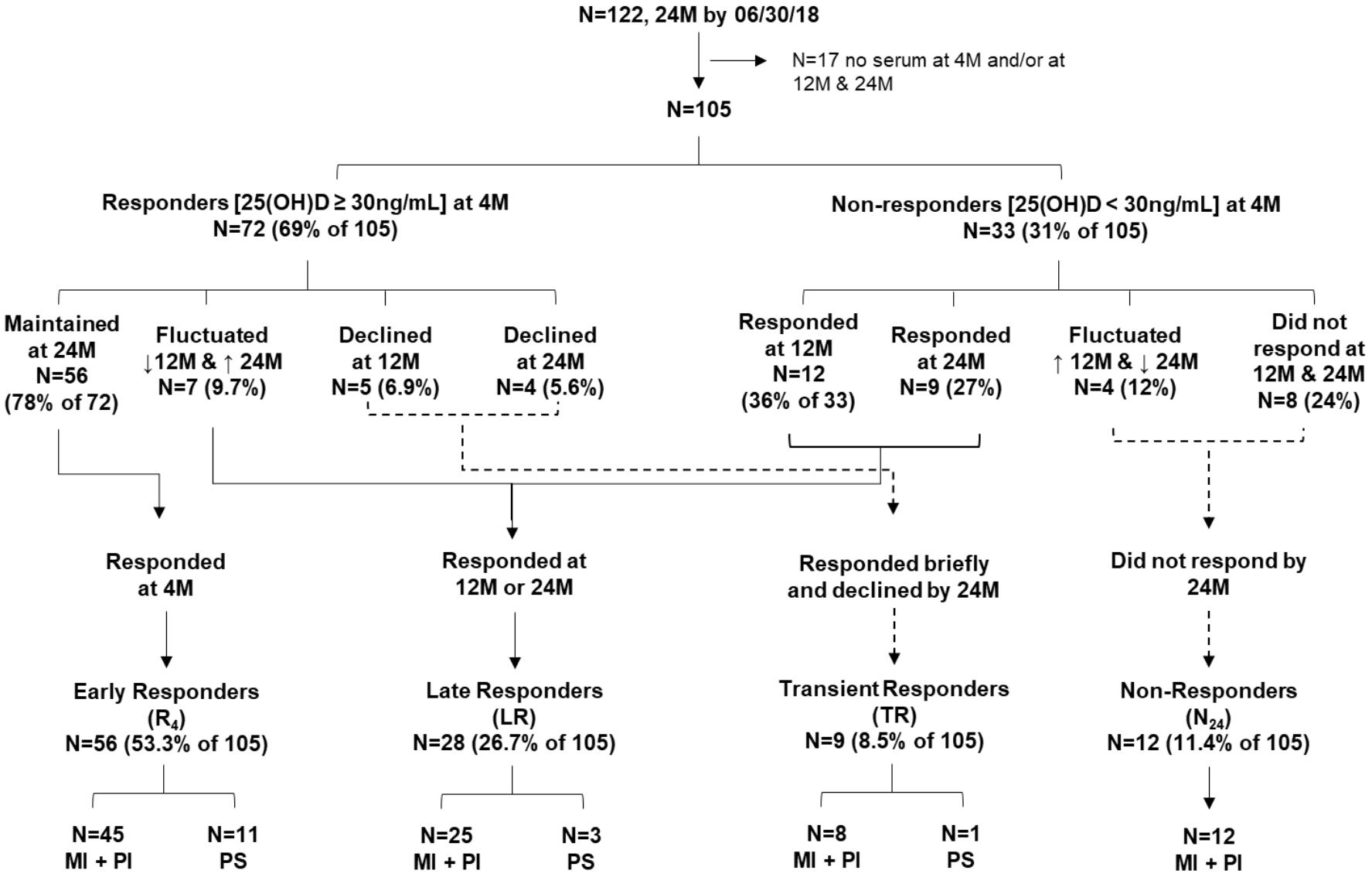
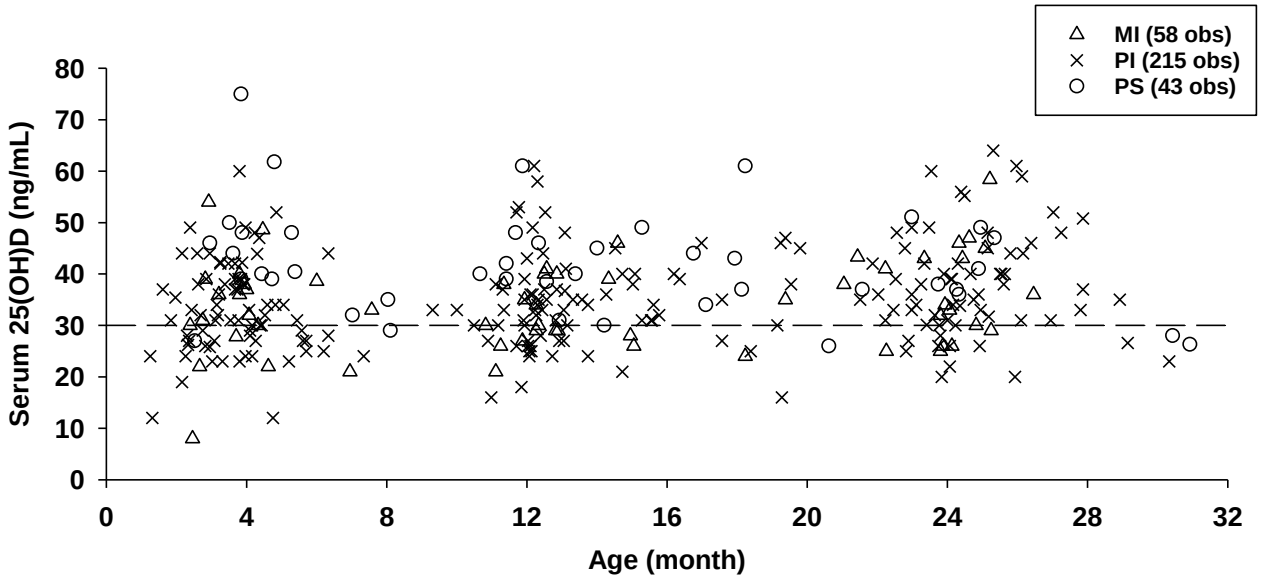


Figure 1. Study population and classification of serum 25-hydroxy vitamin D [25(OH)D] responder status. N=105 included subjects who were PS and excluded subjects who did not have a baseline serum 25(OH)D measured.

A.


MI (58 obs)
PI (215 obs)
PS (43 obs)
Serum 25(OH)D (ng/mL)
0
10
20
30
40
50
60
70
80
0
4
8
12
16
20
24
28
32
Age (month)

B.

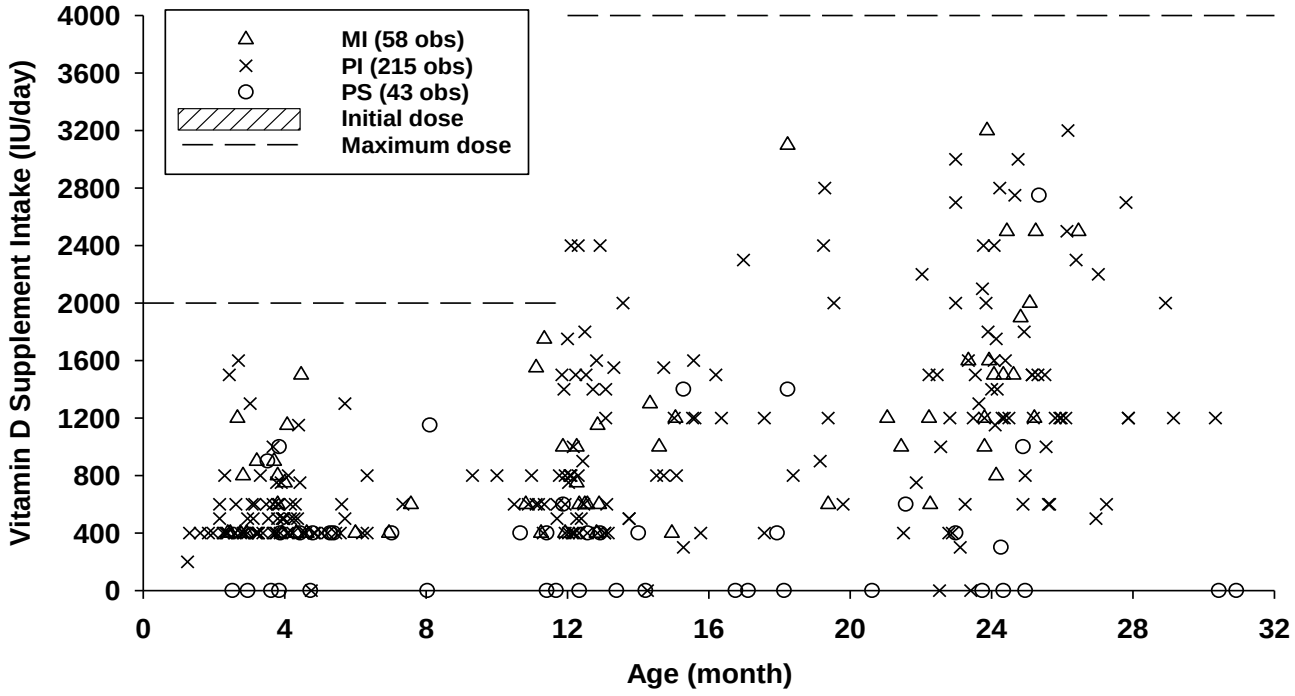

MI (58 obs)
PI (215 obs)
PS (43 obs)
Initial dose
Maximum dose
Vitamin D Supplement Intake (IU/day)
0
400
800
1200
1600
2000
2400
2800
3200
3600
4000
0
4
8
12
16
20
24
28
32
Age (month)

Figure 2. (A) Serum 25(OH)D concentrations in the first 2 years of life by GI phenotype. Dotted line is the cut-off for serum 25(OH)D for vitamin D insufficiency, i.e. <30ng/mL. (B) Vitamin D supplement intake in the first 2 years of life by GI phenotype. Shaded boxes refer to initial recommended dose at each age group (first year of life and second year of life) and dotted lines refer to the maximum dose at each age group.

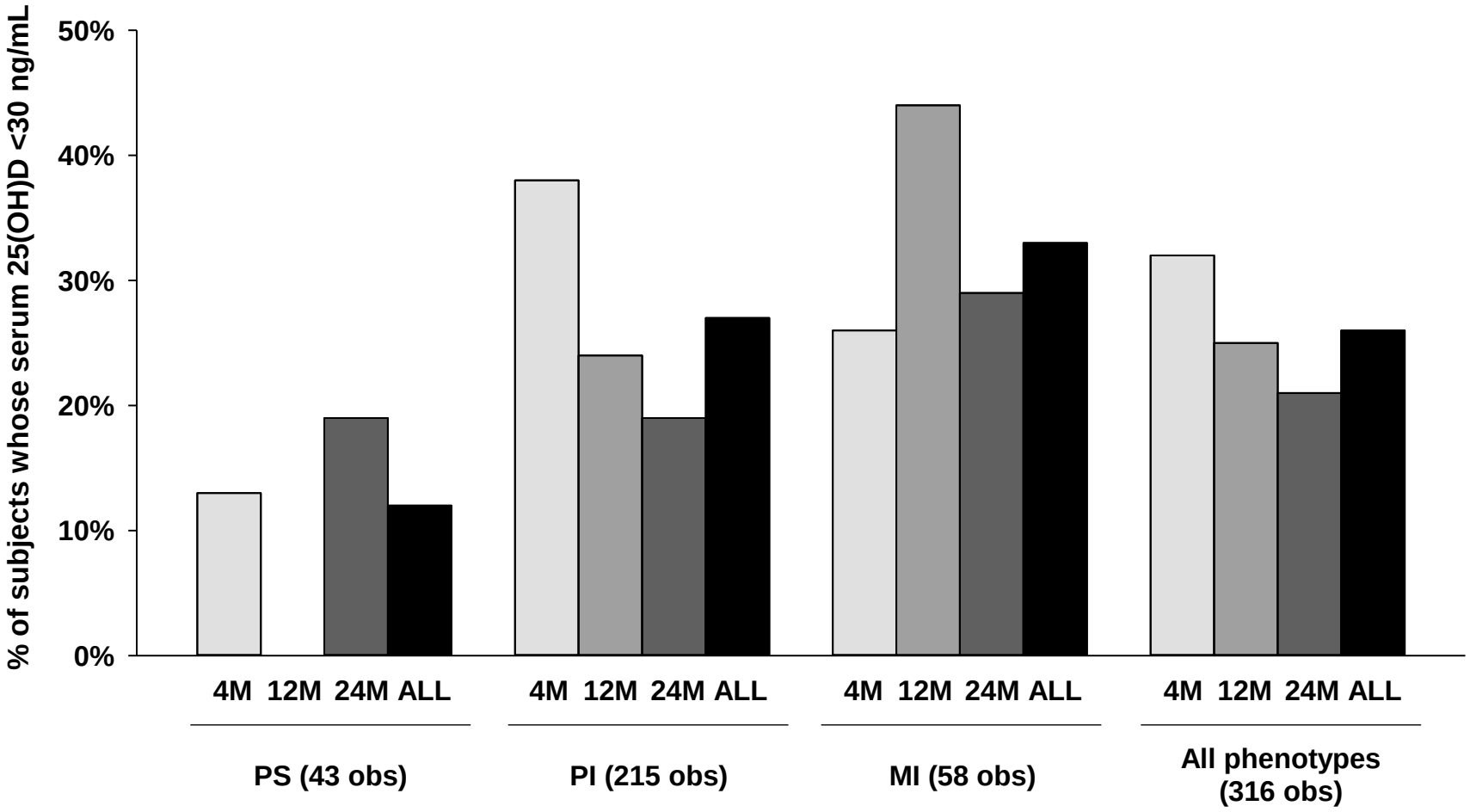


Figure 3. Percent of subjects who were vitamin D insufficient (serum 25(OH)D <30ng/mL) at each time point by GI phenotype.

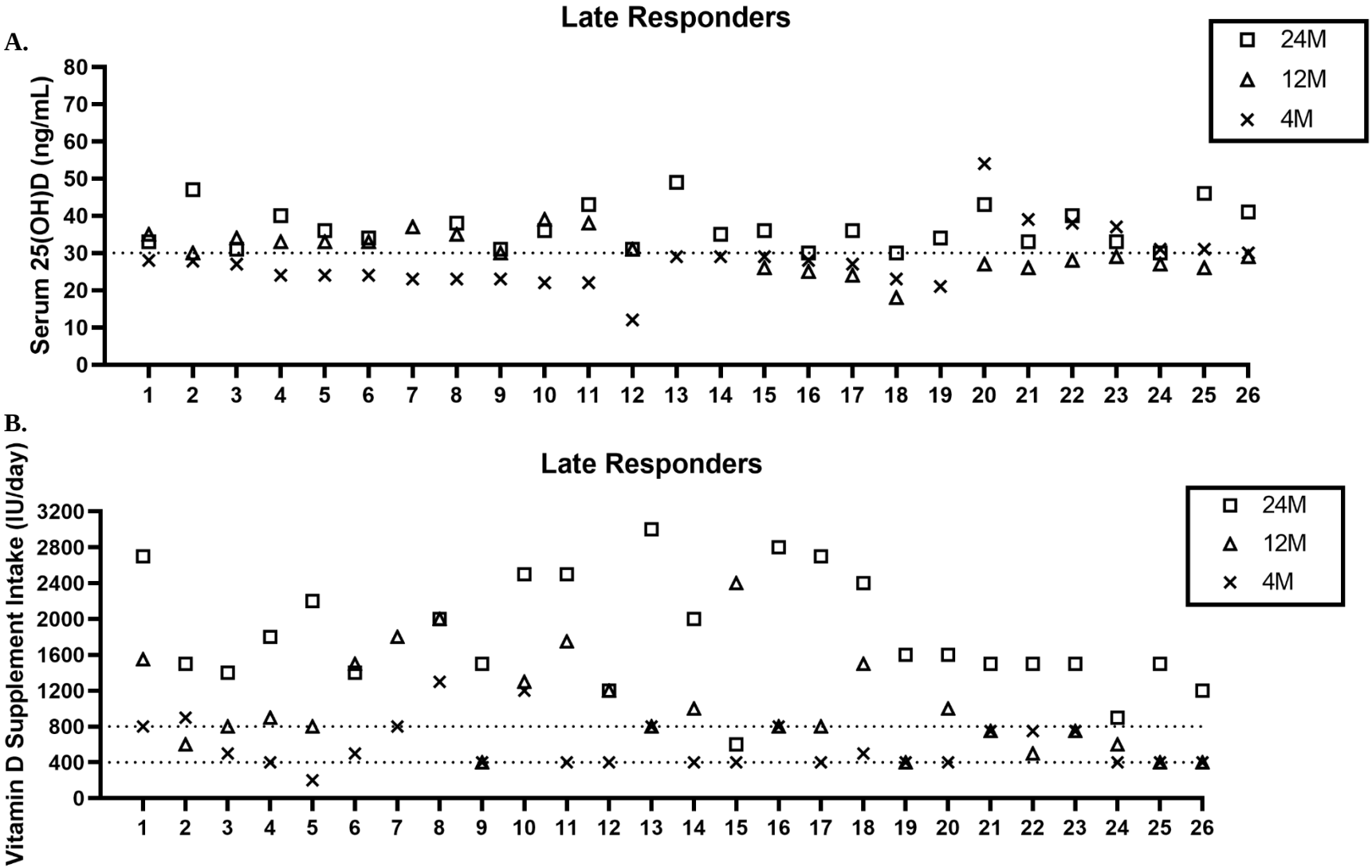


Figure 4. Individual serum 25(OH)D concentrations in the first 2 years of life by subject in (A) late responders (LR); dotted line refers to the cut-off for serum 25(OH)D for vitamin D insufficiency, i.e. <30 ng/mL. Individual vitamin D supplement intake in the first 2 years of life by subject in late responders (LR); dotted line refers to the initial recommended intake for vitamin D supplement at each age group (first year of life and second year of life).

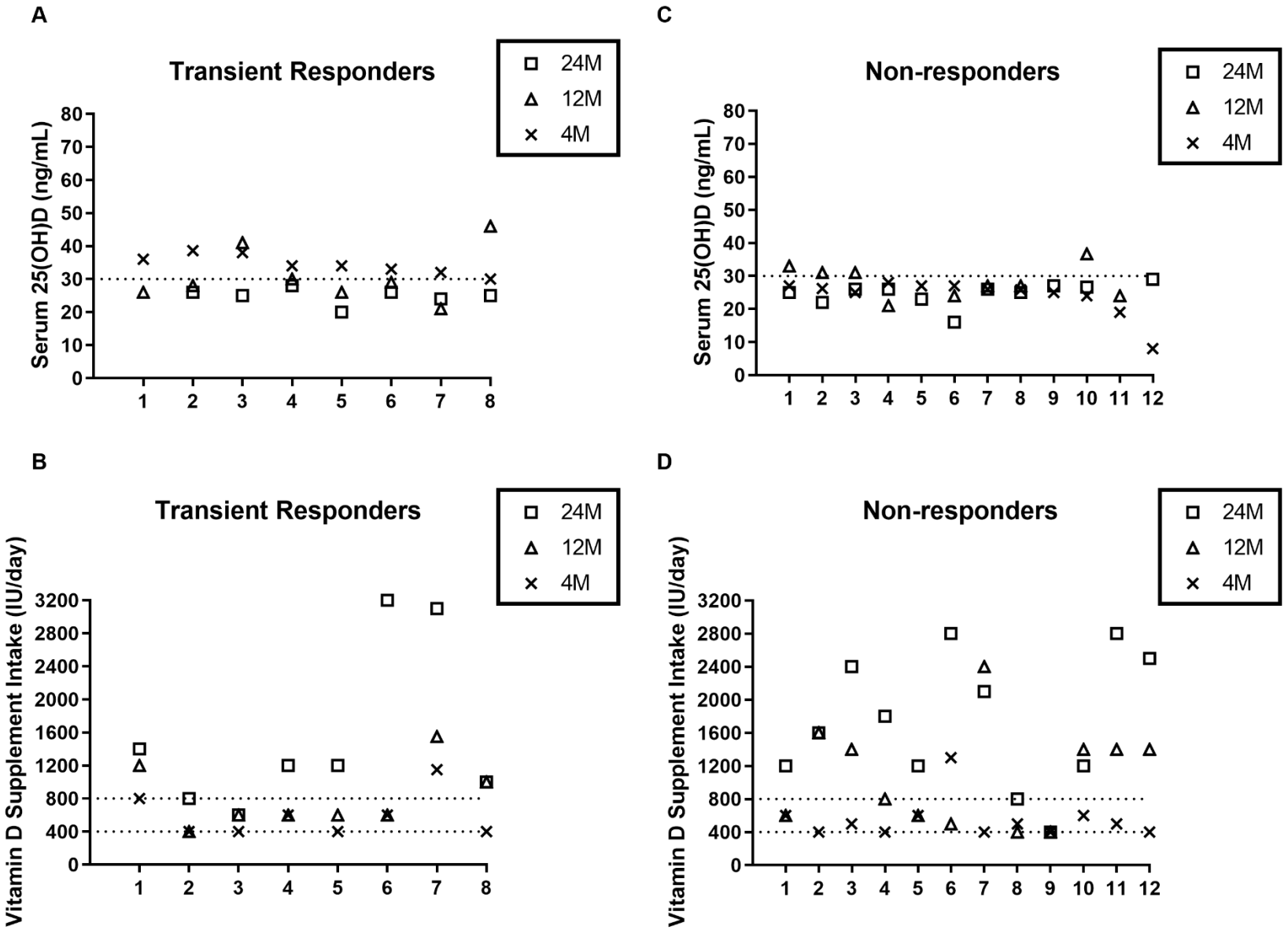
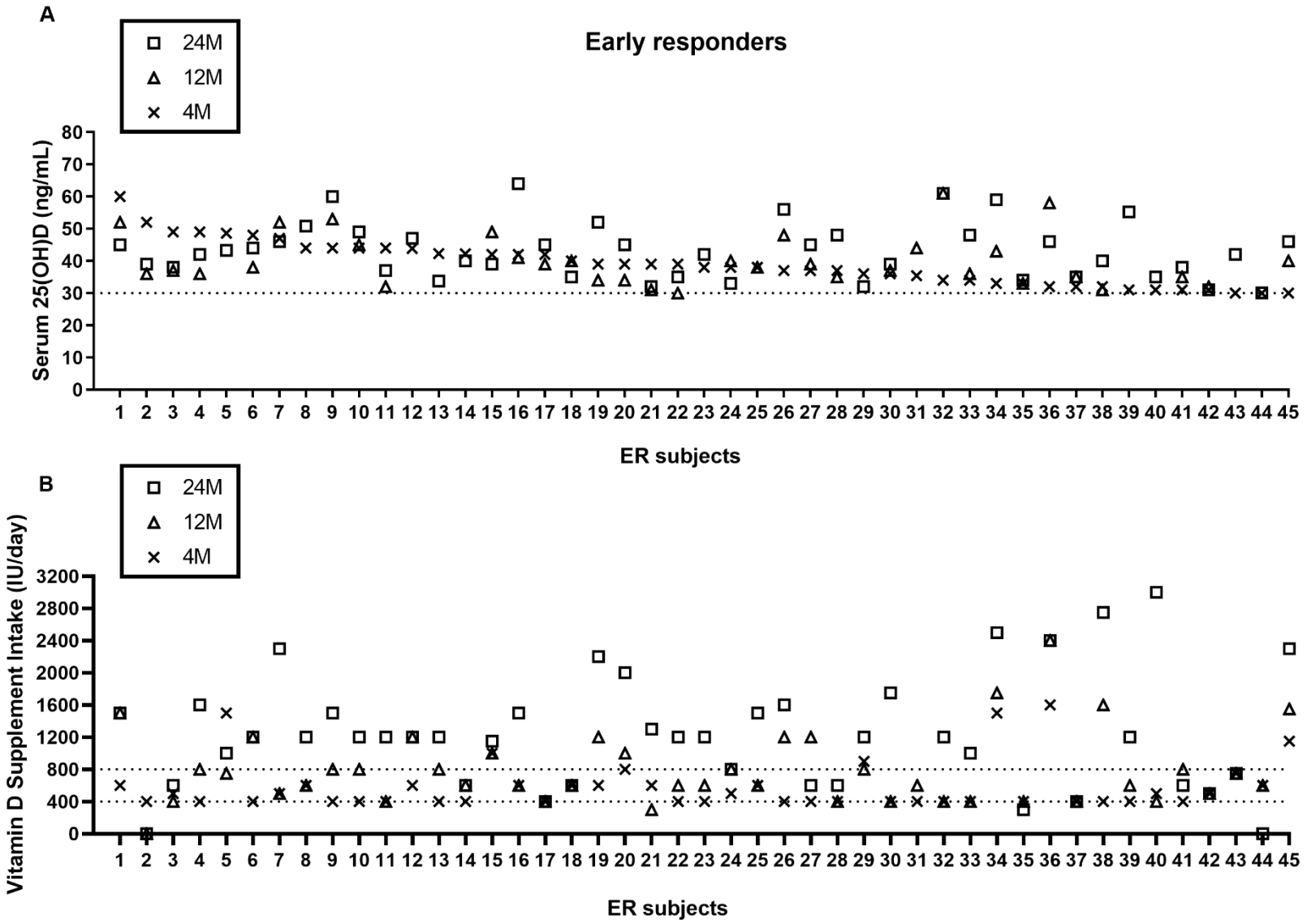

A
Transient Responders
24M
12M
4M
Serum 25(OH)D (ng/mL)
0
10
20
30
40
50
60
70
80
1
2
3
4
5
6
7
8
C
Non-responders
24M
12M
4M
Serum 25(OH)D (ng/mL)
0
10
20
30
40
50
60
70
80
1
2
3
4
5
6
7
8
9
10
11
12
B
Transient Responders
24M
12M
4M
Vitamin D Supplement Intake (IU/day)
0
400
800
1200
1600
2000
2400
2800
3200
1
2
3
4
5
6
7
8
D
Non-responders
24M
12M
4M
Vitamin D Supplement Intake (IU/day)
0
400
800
1200
1600
2000
2400
2800
3200
1
2
3
4
5
6
7
8
9
10
11
12

Figure 5. Individual serum 25(OH)D concentrations in the first 2 years of life by subject in (A) transient responders (TR) and (C) non-responders (NR); dotted line refers to the cut-off for serum 25(OH)D for vitamin D insufficiency, i.e. <30 ng/mL. Individual vitamin D supplement intake in the first 2 years of life by subject in (B) transient responders (TR) and (D) non-responders (NR); dotted line refers to the initial recommended intake for vitamin D supplement at each age group (first year of life and second year of life).



Supplementary Figure 1. (A) Individual serum 25(OH)D concentrations in the first 2 years of life by subject in early responders (ER). (B) Individual vitamin D supplement intake in the first 2 years of life by subject in early responders (ER).

CHAPTER 3

COMPARISON OF 25(OH)D CONCENTRATIONS AMONG CF CENTERS AND IMPLICATIONS FOR VITAMIN D STATUS INTERPRETATION

Lyanne H. Chin, BSc, RD[1], Sangita G. Murali, PhD[1], HuiChuan J. Lai, PhD, RD[1-3], on behalf of the FIRST Study Group

Abstract

Vitamin D insufficiency is a longstanding concern in individuals with CF. Assays to measure 25(OH)D values have varied, leading to non-standardized 25(OH)D values among clinical laboratories. FIRST (*Feeding Infants Right… from the STart*) is a multicenter, prospective, longitudinal cohort study conducted in infants and young children with Cystic Fibrosis to examine their nutritional status up to 6 years of age. The objective of this brief communication is to compare the 25(OH)D values measured in 3 clinical laboratories to the clinical laboratory used for the study. We found that 2 of the 3 sites' serum 25(OH)D concentrations are not significantly different from our study's serum 25(OH)D. The remaining site had 25(OH)D values that were consistently higher than our study's serum 25(OH)D, resulting in a possible misclassification of vitamin D status if local 25(OH)D was used instead. It is therefore imperative for clinicians to interpret 25(OH)D value with caution, bearing in mind within-assay variation, and reacting accordingly to 25(OH)D values that are on the cusp of the cut-off.

INTRODUCTION

Vitamin D deficiency is prevalent in individuals with cystic fibrosis (CF) despite supplementation (1-3). It is recommended by the CF Foundation for all individuals with CF to maintain serum 25-hydroxyvitamin D (25(OH)D) ≥30ng/mL (4). Besides bone health, optimal vitamin D status is also associated with pulmonary health in individuals with CF. Serum 25(OH)D concentration was demonstrated to be strongly positively correlated with forced expiratory volume in one second (FEV_1) % predicted (3) and serum 25(OH)D ≥20 ng/mL in CF has been shown to correlate with fewer episodes of pulmonary exacerbations (1).

The variability in the measurement of 25(OH)D as a result of different assays and lack of standardization have led to varying 25(OH)D values used to define suboptimal vitamin D status and therefore treatment for this deficiency (5). The Vitamin D Standardization Program (VDSP) was developed to standardize 25(OH)D measurements worldwide (6). Our prospective multicenter cohort study presented us with an opportunity to compare 25(OH)D concentrations measured among CLIA-approved clinical laboratories. The objective of this brief communication is to compare 25(OH)D concentrations measured at 3 local clinical laboratories with the clinical laboratory used for the study, allowing for 10% variation and to determine if the vitamin D status agree based on the local 25(OH)D and study 25(OH)D values.

SUBJECTS AND METHODS

Study design and population

The study population consisted of infants born between February 2012 and August 2017, diagnosed through newborn screening, and enrolled in FIRST (*Feeding Infants Right… from the STart*), a multicenter, prospective, observational study conducted at 5 CF centers (Boston, MA, Indianapolis, IN, Salt Lake City, UT, Madison and Milwaukee, WI) to identify optimal feeding for infants with CF. The study was approved by the Institutional Review Boards at the University of Wisconsin-Madison and all participating institutions. Parents of all subjects provided written informed consent. A total of 178 infants born in 2012-2017 were enrolled in the FIRST study at age 1.8 ± 1.0 months (mean ± SD).

Measurement of 25(OH)D concentrations

Blood specimens were obtained at approximately 4 months (4M), 1 year, and annually thereafter up to 6 years of age. Serum from all study specimens was sent to the Clinical Laboratory Services of the University of Wisconsin Hospitals and Clinics within 2 days of receipt for quantification of 25(OH)D concentrations using high-performance liquid chromatography (HPLC) with UV detection (7). Optimal vitamin D status is defined as serum 25(OH)D ≥30 ng/mL, vitamin D insufficient is when serum 25(OH)D is at 20 to 29 ng/mL and serum 25(OH)D <20 ng/mL is defined as vitamin D deficient.

Statistical Analyses

Statistical analyses were performed using SAS (v9.4, SAS Institute Inc., Cary, NC) and data were plotted using GraphPad Prism (v8, GraphPad Software, La Jolla, CA); p values <0.05

were considered as significant. Chi-square test was used to determine the association between 2 categorical variables and Fisher's exact test was used in place of chi-square when the cell sizes were expected to be less than 5. Linear mixed effects model was used to assess the association among 25(OH)D concentration, method, age, and site.

RESULTS AND DISCUSSION

There was a total of 193 blood specimens drawn that were measured both at the UW-Madison and at its local CLIA-approved laboratory for 3 CF centers. **Table 1** showed the characteristics of the blood specimens by sites. In 2 of the 3 sites, there were more blood specimens that were in the less than 2 years old group than in the 2 to 6 years old group. Using absolute serum 25(OH)D values, there were no significant differences among 3 sites for the scenario where study 25(OH)D < 30ng/mL and local 25(OH)D ≥ 30 ng/mL, p=0.289 (Table 1). Similarly, allowing for 10% variation, there were also no significant differences among the 3 sites, p=0.139. This is crucial because there could be a misclassification of suboptimal vitamin D status depending on which value was used and hence "missed" opportunities for increasing vitamin D supplementation.

There was more variation in the differences between local 25(OH)D and study 25(OH)D values for site C, as shown in **Figure 1**. Using a mixed effects model, age as a categorical variable (as in Table 1) was significantly associated with 25(OH)D concentration ($p<0.001$), site was not significantly associated with 25(OH)D concentration ($p=0.975$), and method (local or study) was significantly different where local 25(OH)D concentration was significantly higher than study 25(OH)D concentration, $p<0.001$. Excluding site C in our analysis, method was not significantly different between sites A and B ($p=0.132$), age remained significantly associated with 25(OH)D concentration ($p<0.001$) and site was not ($p=0.806$).

Due to variation in assays used to quantify 25(OH)D, the "true" value is not known. The VDSP guidance allows up to 10% CV, i.e. if the reported 25(OH)D concentration is 20ng/mL, the true value of the 25(OH)D concentration is within the range of 16 ng/mL to 24 ng/mL (5). Correlation is misleading because when 2 methods are meant to measure the same biomarker, the

correlation of the 2 methods will be high and a high correlation does not necessarily mean that the 2 methods agree with each other (8). As such, it is imperative for clinicians to consider the "true" value of 25(OH)D when it comes to interpretation of vitamin D status. For example, if an individual with CF has a serum 25(OH)D concentration of 32 ng/mL, the individual may appear to have optimal vitamin D status but in reality, this individual may have a "true" 25(OH)D value less than 30 ng/mL which prompts a reactive approach such as an increase in vitamin D supplementation to increase the individual's 25(OH)D concentration.

Overall, this study presented an opportunity to investigate the differences in methodologies in 3 CF centers compared to the study clinical laboratory. There were no significant differences in 25(OH)D values for sites A and B; site C had 25(OH)D values consistently higher than that of study values. As such, there is a higher possible misclassification of vitamin D status with site C depending on which value was used. For the purpose of our research, since the study 25(OH)D values are used when available, there is little concern for overestimation of the "true" value of 25(OH)D. However, in practice, it will be crucial for clinicians to not overlook the possible interpretation of 25(OH)D concentrations especially when the values are at the cusp of the cut-offs.

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Table 1. Characteristics of 25(OH)D concentrations used for comparison by site

	Site A	Site B	Site C	p
N analyzed (pairs)	40	69	84	-
Absolute 25(OH)D values				
Study <30ng/mL, Local ≥30ng/mL	4 (10.0%)	6 (8.7%)	14 (16.7%)	0.289
Allowing for 10% variation				
Study <30ng/mL, Local ≥30ng/mL	0 (0%)	3 (4.3%)	7 (8.3%)	0.139
By age groups				
< 2 years	23 (57.5%)	43 (62.3%)	33 (39.3%)	
Absolute 25(OH)D values Study <30ng/mL, Local ≥30ng/mL	4 (10.0%)	5 (7.2%)	6 (7.1%)	0.691
Allowing for 10% variation Study <30ng/mL, Local ≥30ng/mL	0 (0%)	2 (2.9%)	4 (4.8%)	0.192
2 to 6 years	17 (42.5%)	26 (37.7%)	51 (60.7%)	
Absolute 25(OH)D values Study <30ng/mL, Local ≥30ng/mL	0 (0%)	1 (1.4%)	8 (9.5%)	0.161
Allowing for 10% variation Study <30ng/mL, Local ≥30ng/mL	0 (0%)	1 (1.4%)	3 (3.6%)	0.815

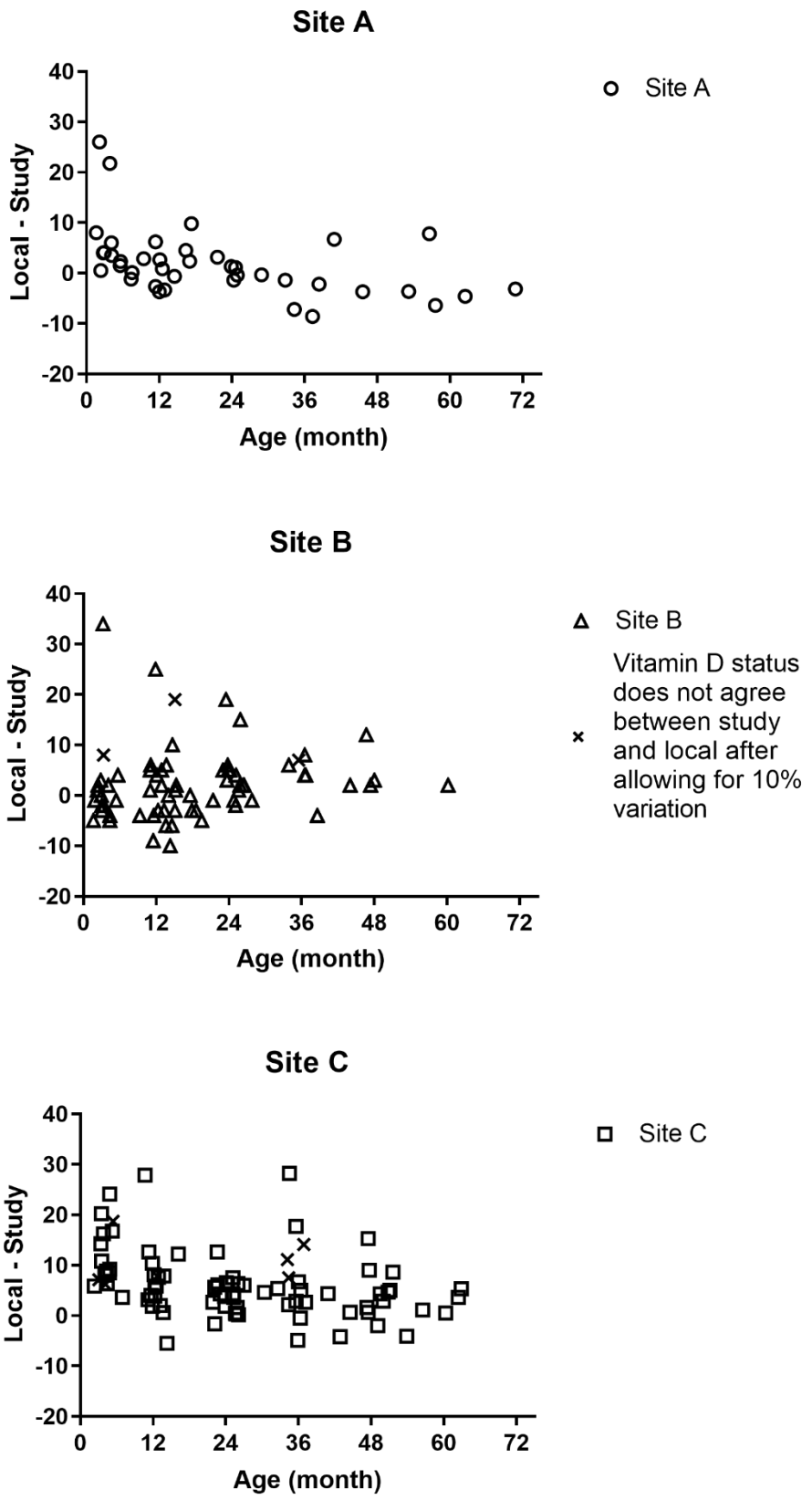

Figure 1. Differences in local and study 25(OH) concentrations by site up to 6 years of age

CHAPTER 4

ESSENTIAL FATTY ACID ABNORMALITIES ARE PREVALENT IN INFANTS AND YOUNG CHILDREN WITH CYSTIC FIBROSIS IN THE FIRST 2 YEARS OF LIFE

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Abstract

Background: Essential fatty acid (EFA) abnormalities have been commonly reported in the CF population. It has been found that EFA abnormalities in CF were observed in well-nourished subjects as well as subjects with pancreatic sufficiency, indicating that there is a basic defect in fatty acid metabolism with CF.

Objectives: To determine the prevalence of EFA abnormalities in infants and toddlers with CF diagnosed via newborn screening, investigate the variation in fatty acid profiles in these children, and explore the association between dietary fat intake and major fatty acids.

Design: Data from an ongoing multicenter prospective longitudinal observational study initiated in 2012 at 5 CF Centers in the US to identify optimal feeding for infants with CF were analyzed. Fatty acids from red blood cells were quantified at 3 time points in the first 2 years of life and dietary intakes were assessed using 3-day food records.

Results: The prevalence of EFA abnormalities increased with age in the first 2 years of life; about 50% of our cohort were EFA deficient or insufficient at 2 years of age. Subjects who were EFA deficient had lower linoleic acid concentrations than subjects who were EFA sufficient. Overall, we identified 4 patterns of responsiveness to EFA status; half of our cohort were early or late responders and the other half were transient or non-responders. Meconium ileus is a significant predictor for EFA responder status. In addition, our cohort consumed 30% more in terms of calories but 80% were still not meeting the dietary requirements for linoleic acid at 2 years of age.

Conclusion: Given the high prevalence of EFA abnormalities, EFA status should be monitored routinely even in the absence of EFA deficiency symptoms.

Keywords: cystic fibrosis, essential fatty acid status, linoleic acid, triene:tetraene

INTRODUCTION

Essential fatty acid (EFA) abnormalities have been a longstanding concern in individuals with cystic fibrosis (CF) (1-3). What was initially thought to be secondary to intestinal fat malabsorption is now proven to be inaccurate; these EFA abnormalities were also reported in individuals who were pancreatic sufficient (PS), suggesting that the abnormalities are not just a consequence of intestinal fat malabsorption (4). Fatty acid abnormalities that were observed in individuals with CF and in animal models indicate that there are fundamental changes in the metabolism of fatty acids that occur in cells of patients with CF (5, 6). The most commonly reported essential fatty acid abnormalities are a decrease in linoleic acid (LA, 18:2n-6) and docosahexaenoic acid (DHA, 22:6n-3) concentrations (3, 7, 8), increased arachidonic acid (AA, 20:4n-6) concentrations (7, 8), an increase in triene (mead acid)-to-tetraene (arachidonic acid) ratio (T:T) (9-11), a biomarker for EFA deficiency (12), as well as increased pathologic triene (mead acid, 20:3n-9) concentrations compared to healthy controls (10). Currently, routine testing for EFA abnormalities is not a recommendation in the infant care guidelines and there is insufficient evidence to recommend for or against supplementation with LA or DHA for nutritional management of infants with CF (13).

Nationwide newborn screening (NBS) in 2010 has allowed for earlier diagnosis of CF and hence intervention for nutritional deficiencies. This is significant clinically as historically infants were presented to the CF clinic with failure to thrive before intervention for nutritional deficiencies were started. EFA abnormalities in CF were reported as early as in the 1960s and undernutrition and pancreatic insufficiency were frequently associated with EFA deficiency (14, 15). However, it was later observed that EFA deficiency exists in well-nourished patients with CF as well as patients who were pancreatic sufficient, which suggested that EFA abnormalities

in CF are more than just malnutrition and malabsorption (1, 4, 16). The present study is significant as it is one of the few multicenter prospective cohort studies that followed infants starting from diagnosis in the post-NBS era. Hence, our findings will be clinically relevant as they are based on current practice in CF centers.

There are few studies that have looked at EFA abnormalities in the pediatric CF population (7, 9, 11, 17). A cross-sectional study that was conducted in 13 CF centers looked at the dietary intake in children ages 7 to 10 years and found that total dietary fat intake was positively associated with serum LA concentrations and inversely with T:T, which indicated that fat intake had a significant role in increasing serum LA concentrations (11). Another study conducted in Wisconsin showed better growth in infants with CF who consumed a predigested formula that contained high LA (12% energy) compared to those who consumed a comparable formula with lower LA (7% energy), despite a lower intake of total energy in the former group (17). However, no longitudinal studies regarding EFA status in CF have been published in the past few years. As such, our multicenter prospective longitudinal study plays a critical role in adding evidence to the current field regarding EFA status in infants with CF.

Our present cohort study presented an opportunity to explore EFA abnormalities in infants under the current clinical care for infants with CF in the first 2 years of life. The objectives of this paper are to determine the prevalence of EFA abnormalities in infants and toddlers with CF diagnosed via NBS, investigate the variation in fatty acid profiles in these children, and explore the association between dietary fat intake and major fatty acids.

SUBJECTS AND METHODS

Study design and population

The FIRST (*Feeding Infants Right… from the STart*) is a multicenter, prospective, observational study conducted at 5 CF centers to identify optimal feeding for infants with CF. Details of the study have been described previously (see Chapter 2). Of the 178 subjects enrolled in the study, 141 subjects were at least 24 months of age by 06/30/2018. Five infants withdrew before 3 months of age, 9 had a low birth weight (<2500 g), and another 6 did not have red blood cell (RBC) specimens in the first 2 years of life; the final analyses were done on 121 subjects **(Figure 1)**.

Assessment of fatty acid intake

Nutritional and pulmonary questions were collected at each routine clinic visit in the first 2 years of life. A 3-day food diary was completed by parents prior to the subject's 24-month clinic visit; the food diaries were included for analyses if the dates fell within 3 months of the 24-month blood draw. The food diaries were analyzed using Food Processor Nutrition Analysis Software (ESHA Research, Salem, OR).

Infants' feeding history in the first 6 months of life were classified into 2 broad categories – unfortified feedings and fortified feedings. Unfortified feedings refer to exclusive breastmilk, exclusive formula at the standard caloric density (20 kcal/oz), or breastmilk and formula at 20 kcal/oz for 6 months. Fortified feedings refer to formula mixed to 22+ kcal/oz, breastmilk with formula added in the same bottle, or any combination of breastmilk and formula that were at a higher caloric density, i.e. greater than 20 kcal/oz.

Laboratory analyses

Blood specimens were obtained at approximately 4 months (4M), 12 months (12M), and 18 to 24 months (24M) of age. Fatty acids were measured in RBC by gas liquid chromatography (Agilent 6890 Series, Santa Clara, CA) with a flame ionization detector. We modified a simplified method for quantitative analysis of fatty acids with the addition of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) as internal controls (18). Boron trifluoride (BF_3) in 14% methanol (Sigma-Aldrich, St. Louis, MO) was added directly to the homogenized samples for methylation, followed by extraction with hexane. Chromatograms were analyzed using HP ChemStation Software. Results were normalized to C15:0 and each fatty acid was expressed as a weight percent of total identified fatty acids in RBC. A total of 290 RBC specimens from 121 subjects were analyzed.

Classification of EFA status and response to EFA status

T:T was used to define EFA status from RBC specimens since they represent long term storage (19). EFA deficiency (EFAD) was defined as T:T >0.02, EFA insufficiency (EFAI) was defined as 0.01< T:T ≤0.02, and EFA sufficiency (EFAS) was defined as T:T ≤0.01.

EFA status was classified based on the subject's response to clinical treatments for CF in the first 2 years of life. Since the purpose of the study was to investigate EFA status in the first 2 years of life, only subjects who had a baseline, i.e. around 4M, blood specimen collected and with at least 1 follow-up blood specimen collected at 12M or 24M were included. Of 121 subjects, 16 subjects only had blood collected once in the first 2 years of life and another 16 subjects did not have a baseline blood specimen, the remaining 89 subjects were then classified into various EFA status based on their overall response (Figure 1).

Early responders (ER, n=28) were subjects who were EFA sufficient at baseline and maintained at 24M. Late responders (LR, n=17) were subjects who were either EFA insufficient or deficient at baseline and became EFA sufficient at 12M or 24M or were EFA sufficient at baseline, declined at 12M, and became EFA sufficient again at 24M. Transient responders (TR, n=31) were subjects who were EFA sufficient at 4M and/or 12M and became EFA insufficient or EFA deficient at 12M or 24M. Non-responders (NR, n=13) were subjects who were EFA insufficient or deficient throughout the first 2 years of life or were EFA insufficient or deficient at baseline, improved to EFA sufficient at 12M, and declined to EFA insufficient or deficient at 24M.

Statistical analyses

Statistical analyses were performed using SAS (v9.4, SAS Institute Inc., Cary, NC); data were plotted using GraphPad Prism (v8, GraphPad Software, La Jolla, CA). Data were presented as mean ± SD unless otherwise stated; p values <0.05 were considered as significant. Data were checked for normality using Shapiro-Wilk test; if data were not normally distributed, data were transformed to normality whenever possible or a non-parametric test was used. Chi-square test was used to determine the association between 2 categorical variables and Fisher's exact test was used in place of chi-square when the cell sizes were expected to be less than 5. T-test and Mann-Whitney U test, where applicable, were used to determine whether the means of 2 groups differ significantly from each other. ANOVA and Kruskal-Wallis (where applicable) were used to compare differences among group means and medians, respectively. Linear regression models were used to explore the association between fatty acid concentrations, phenotype, and age.

Logistic regression was used to assess the association between responder groups, using sex and phenotype categories as covariates.

RESULTS

Characteristics of study population

A brief description of the characteristics of the 121 subjects was described in **Table 1** according to their EFA status. Overall, 68.6% of the subjects were pancreatic insufficient (PI), 54.5% were male, and 48.7% were F508del homozygotes.

Prevalence of essential fatty acid abnormalities

The prevalence of EFA abnormalities (insufficiency and deficiency) were 31% (9.5% EFAI, 21.5% EFAD) at 4M, 35% (15% EFAI, 20% EFAD) at 12M, and 48% (36.5% EFAI, 11.5% EFAD) at 24M, as shown in **Figure 2**. The prevalence of EFA deficiency and insufficiency were higher at 24M compared to 4M and 12M, $p<0.001$. We looked at the prevalence of EFA deficiency and insufficiency for the 3 GI phenotypes at various time points in the first 2 years of life and found that at 12M, subjects with PI tended to have a higher prevalence of EFA abnormalities than subjects with PS, $p=0.058$; no significant differences were observed at 4M and 24M (Figure 2). The T:T in the first 2 years of life was not significantly different with age or phenotype (**Figure 3**).

Red blood cell fatty acid profile in the first 2 years of life

LA and eicosapentaenoic acid (EPA, 20:5n-3) concentrations in RBC increased with age ($p<0.001$ and $p=0.002$, respectively) whereas AA and DHA concentrations decreased with age, $p<0.001$. AA concentrations were also significantly higher in subjects who were PS compared to subjects who were PI or had MI, $p=0.006$. ALA and mead acid concentrations did not change significantly with age or phenotype (**Figure 4**).

At baseline, subjects who were EFAD had significantly lower AA concentrations than subjects who were EFAS, p=0.023, as shown in **Figure 5**. Similarly, those who were EFAD and EFAI had significantly lower LA concentrations than those who were EFAS, p=0.004. At 12M, subjects who were EFAD had significantly lower AA concentrations and subjects who were EFAI had significantly lower LA concentrations than subjects who were EFAS, p=0.022 and p=0.003, respectively. At 24M, there were no significant differences in LA concentrations among the EFA status and subjects who were EFAD had significantly lower AA concentrations compared to subjects who were EFAI and EFAS, p=0.007.

Table 2 showed selected fatty acid concentrations at each time point. With the exception of ALA concentrations and T:T, most fatty acid concentrations were significantly different at each time point. The ratio of AA to LA decreased significantly from 4M to 12M, whereas the ratio of AA to DHA increased significantly from 12M to 24M, both p<0.001. The ratio of LA to dihommo-gammalinolenic acid (DGLA, 20:3n-6), an indirect marker of Δ6-desaturase activity, was found to be higher in 12M than in 4M and 24M, p<0.001.

EFA responder status in the first 2 years of life

About 50% of our cohort were responders, either early (31.5%) or late (18.0%), and another 50% of our cohort were transient (34.8%) and non-responders (15.7%, Figure 1). There were similar rates of infections, hospitalizations, and *Pseudomonas* colonization among the EFA responder status. Phenotype was significantly associated with EFA responder status, p=0.048; subjects who had MI were less likely to be in the ER group compared to subjects who were PI (OR: 0.279, 95% CI: 0.089-0.875).

Type of feeding and EFA abnormalities at 4M

Within the unfortified feeding groups, those who were exclusively breastfed for 6 months had the highest prevalence of EFAD and EFAI at 12M and 24M, p=0.064 and p=0.062, respectively (**Figure 6A**). The prevalence of EFA abnormalities were 67% and 79% at 12M and 24M, respectively, for subjects who were exclusively breastfed. At baseline, those who were exclusively breastfed had lower LA concentrations and higher EPA concentrations than those who were on exclusive formula and on both breastmilk and formula, p<0.001 and p=0.005, respectively. ALA, AA, and DHA concentrations were not significantly different among the 3 unfortified feeding groups at 4M of age.

Within the fortified feeding groups, the feeding groups were categorized by time of the start of fortification, i.e. at 1 month, 2 months, or 3 months and beyond. The EFA status distribution was not significantly different among the fortified feeding groups at each time point (**Figure 6B**). LA, ALA, AA, EPA, and DHA concentrations were not significantly different among the 3 fortified feeding groups at baseline.

LA, ALA, EPA, and DHA concentrations were not significantly different among subjects who were on fortified feedings and subjects who were on unfortified feedings (data not shown). Only AA concentrations were lower in subjects who were on fortified feedings than subjects who were on unfortified feedings, p=0.038.

Dietary intake and association with fatty acids at 24M

About half (52.9%) of the specimens measured at 24M had a corresponding 3-day food diary that was included for analysis. Dietary intake at 24M were reported in **Table 3**. None of the intake variables reported in Table 3 was significantly different among the EFA status at 24M;

phenotype was included as a covariate and dietary intake was not significantly different for each phenotype except for protein expressed as % kcal/day, p=0.046. Subjects who were PS consumed significantly higher protein (% kcal/day) than subjects who had MI. Dietary LA expressed as % kcal/day was significantly correlated with DHA (r=0.232, p=0.018) but not with LA (r=0.014, p=0.921) and AA (r=-0.256, p=0.161, **Figure 7**). Dietary PUFA (g/day) was positively correlated with DHA (r=0.274, p=0.043) and dietary MUFA (g/day) was negatively correlated with LA (r=-0.341, p=0.011).

Most of the subjects (80.0%) consumed LA less than the Adequate Intake (AI) of 6.3% kcal/day at 24M even though their caloric intakes were about 30% greater than their estimated energy requirements (**Table 3**). The prevalence of EFAD and EFAI were not significantly different among GI phenotypes stratified by LA intake less than or equal to and greater than the AI. Similarly, the prevalence of EFAD and EFAI were not significantly different among subjects who were PI stratified by LA intake.

DISCUSSION

EFA abnormalities have been frequently reported in the CF population (1, 2, 7). Our study found that the prevalence of EFAD and EFAI increased significantly with age in the first 2 years of life for children with CF (31% at 4M, 35% at 12M, and 48% at 24M). EFA abnormalities were previously thought to be secondary to pancreatic insufficiency, however, these abnormalities were also observed in CF patients who were pancreatic sufficient (4, 20, 21). Our study showed that the prevalence of EFAD and EFAI in subjects who were PS ranged from 8.3% to 41.7% in the first 2 years of life; though at 12M, subjects with PI tended to have a higher prevalence of EFAD and EFAI than subjects with PS (45.3% in PI, 8.3% in PS).

Our study reported selected RBC fatty acid concentrations at 3 time points in the first 2 years of life. This is one of the few studies that has investigated longitudinal EFA status in the recent years. We reported that most fatty acid concentrations changed significantly with age, suggesting that there are changes in the fatty acid composition from infancy to 2 years of age. LA and EPA increased with age whereas AA and DHA decreased with age. The decrease in AA to LA ratio suggested that the flux of n-6 pathway from LA to AA decreased, i.e. less LA is utilized to produce AA with age. The decrease in DHA and increase in LA and EPA with age could be attributed to the competition for Δ-6 desaturase as the conversion of EPA to DHA also utilizes Δ-6 desaturase, hence when more LA is in circulation, less Δ-6 desaturase is used to convert EPA to DHA, resulting in increased EPA and decreased DHA concentrations (22). Yet, a study previously found that LA concentrations decreased with age in CF patients, possibly due to poorer pulmonary function leading to increased oxidative stress and oxidation of LA (10). However, the age range for this study was from 1 to 41 years of age whereas our study is focused on just the first 2 years of life.

AA to DHA ratio was found to be elevated in the nasal biopsy specimens and plasma specimens of CF patients compared to healthy controls (3, 8). Also, it was previously shown that a high AA to DHA ratio was associated with impaired bone mineral density in children CF (23). Our study observed that the ratio of AA to DHA increased from 12M to 24M, which is consistent with the increase in the prevalence of EFA abnormalities.

The most commonly reported EFA abnormality in the CF population is decreased LA concentrations (2, 7, 24). Even though our study does not have a control group, we found that within the CF population, the mean LA concentrations of subjects who were EFAD and EFAI at 4M and subjects who were EFAI at 12M were significantly lower than subjects who were EFAS, which further supported that low LA is another characteristic of EFA abnormality. Likewise, subjects who were EFAD had lower mean AA concentrations at all 3 time points than subjects who were EFAS, which was expected since the EFA status was defined based on the ratio of mead acid to AA. One hypothesis behind low LA in circulation is that there is an increase in the metabolic conversion of LA to its downstream metabolites such as AA (25); however, in our study, we found that those who were EFA deficient tended to have lower LA and AA concentrations. AA is indispensable for brain growth especially during infancy (26); lowered AA concentrations observed could have potential effects in the development of the brain of children who were EFA deficient.

Furthermore, we identified 4 main patterns of responses to EFA status, i.e. early responders, late responders, transient responders, and non-responders. About half of our cohort are responders (early or late) and the other half are transient or non-responders. Phenotype was the only variable that was significantly associated with EFA responder status; having a history of MI reduced the odds of being in the ER group, which was expected given that the nutritional

status of those with MI tended to be poorer than subjects who were PI (27). Given that children with CF are not actively supplemented with EFA, it is not surprising that half of our cohort declined in their EFA status in the first 2 years of life and that half of our cohort had EFAD or EFAI at 2 years of age.

Additionally, our study investigated the association between dietary intake and EFA status. Infants who were exclusively breastfed had the highest prevalence of EFAD and EFAI compared to infants who were on unfortified formula or unfortified mixed feedings at all 3 time points. This could be attributed to the varied EFA (LA and ALA) content in breastmilk, which is dependent on maternal intake (28, 29) even though a study had compared various infant formulas on the market and found no differences between LA content in breastmilk and infant formulas (30). However, it has been reported that the conversion of LA and ALA to their downstream metabolites such as AA and DHA are not efficient in infants (26). Furthermore, at baseline, breastmilk and/or formula are the only source of nutrients for infants, hence, most will have adequate LA intake.

At 24M, dietary intake was not found to be significantly associated with EFA status. One explanation for the lack of association could be that only half of subjects who had blood drawn at 24M filled out the 3-day food diary that was within 3 months of the blood draw. In contrast to our findings, a study found that dietary intake was significantly associated with serum phospholipid fatty acids concentrations; this could be because the serum specimens were non-fasting (11) and thus our study measured fatty acids in RBC which would signify long term storage since getting fasting specimens in the first 2 years of life is not possible.

Interestingly, our study found that at 24M, the children were consuming 30% more in terms of calories compared to their estimated energy requirements, yet most of them (80%)

consumed LA less than the AI (6.3% kcal/day) at 24M. Even with the use of pancreatic enzymes, there is a 15% fat malabsorption in the CF population (31). This means that the AI for children with CF should be 15% more than 6.3% kcal/day and that there were more children in our cohort who were consuming less than 15% more than the AI. Therefore, this could explain the increasing prevalence of EFA abnormalities from 4M to 24M.

Studies have shown that EFA supplementation has some benefits in the CF population. A previous study has shown that an infant formula that contained a higher LA content (12% energy) compared to a lower LA content (7% kcal) was significantly associated with better growth outcomes in infants with CF (17). Comparatively, a study that gave small doses of n-6 and n-3 EFA (LA, gamma-linolenic acid (GLA, 18:3n-6), EPA, and DHA) for a year in adolescents with CF found that their palmitoleic acid (16:1n-7) and AA concentrations decreased compared to baseline and DHA and LA increased compared to baseline and observed improvements in respiratory parameters such as decreased number of exacerbations (32). These studies showed that EFA supplementation may be able to normalize fatty acid composition and improve clinical parameters in patients with CF.

According to the current CFF guidelines, there is insufficient evidence to recommend for or against LA or DHA supplementation in infants with CF in the first 2 years of life and EFA status is not monitored routinely (13). Given the high prevalence of EFA abnormalities observed in our cohort in just the first 2 years of life, it is recommended that EFA status should be monitored even when the child shows no signs and symptoms of EFA deficiency such as dry, scaly skin.

CONCLUSION

Overall, we showed that EFA abnormalities are common in CF patients in the first 2 years of life. Our study described age-related fatty acid changes and compared fatty acid concentrations among subjects with different EFA status. We also found that subjects who had MI were less likely to be an early responder in terms of EFA status. Dietary intake was not significantly associated with RBC fatty acid concentrations and most children at 2 years of age had low linoleic acid intake despite a higher caloric intake. In view of these findings, even in the absence of symptoms of EFA deficiency, EFA status should be monitored routinely in the CF population.

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Table 1. Characteristics of study population (n=121), born between February 2012 and June 2016[1]

		By Essential Fatty Acid Status[2]				
	All (n=121)	**Early Responders (ER) (n=28)**	**Late Responders (LR) (n=17)**	**Transient Responders (TR) (n=31)**	**Non-Responders (NR) (n=13)**	**Not classified (n=32)**
Male	66	17 (25.8%)	8 (12.1%)	16 (24.2%)	7 (10.6%)	18 (27.2%)
Racial/ethnicity categories						
Non-Hispanic White	112	26 (23.2%)	17 (15.2%)	29 (25.9%)	13 (11.6%)	27 (24.1%)
Other[3]	9	2 (22.2%)	0 (0%)	2 (22.2%)	0 (0%)	5 (55.6%)
Diagnosis						
Newborn screening	108	25 (23.1%)	15 (13.9%)	27 (25.0%)	10 (9.3%)	31 (28.7%)
Prenatal screening	13	3 (23.1%)	2 (15.4%)	4 (30.8%)	3 (23.1%)	1 (7.7%)
GI phenotype						
MI	22	7 (31.2%)	2 (9.1%)	4 (18.2%)	0 (0%)	9 (40.9%)
PI	83	16 (19.3%)	12 (14.5%)	26 (31.3%)	11 (13.3%)	18 (21.7%)
PS	16	5 (31.3%)	3 (18.8%)	1 (6.3%)	2 (12.5%)	5 (31.3%)
Genotype						
F508del/F508del	59	14 (23.8%)	8 (13.6%)	17 (28.8%)	8 (13.6%)	12 (20.3%)
F508del/other	52	12 (23.1%)	9 (17.3%)	13 (25.0%)	3 (5.8%)	15 (28.8%)
Other/other	10	2 (20.0%)	0 (0%)	1 (10.0%)	2 (20.0%)	5 (50.0%)
Age at first CF center visit (month)[4]	0.69[5] ± 0.61	0.62[7] ± 0.40	0.62 ± 0.42	0.68 ± 0.56	0.64 ± 0.37	0.68 ± 0.37
	0.49[6]	0.51[7]	0.56	0.46	0.49	0.52
Upper respiratory tract infections[8]						
0-1	34	7 (20.6%)	5 (14.7%)	9 (26.5%)	3 (8.8%)	10 (29.4%)
≥2	87	21 (24.1%)	12 (13.8%)	22 (25.3%)	10 (11.5%)	22 (25.3%)
Hospitalizations[9]						
None	66	17 (25.8%)	9 (13.6%)	18 (27.3%)	5 (7.6%)	17 (25.8%)
≥1	55	11 (20.0%)	8 (14.5%)	13 (23.6%)	8 (14.5%)	15 (27.3%)
Pseudomonas aeruginosa acquisition[10]						
None	98	23 (23.5%)	15 (15.3%)	26 (26.5%)	11 (11.2%)	23 (23.5%)
≥1	23	5 (21.7%)	2 (8.7%)	5 (21.7%)	2 (8.7%)	9 (39.1%)

[1]Values are *n* (%) for the corresponding row unless otherwise indicated
[2]See Figure 1 for definition and classification of essential fatty acid status
[3]Other includes American Indian or Alaska Native, Asian, Black or African American, Hispanic or Latino, or more than one race according to National Institutes of Health's definitions for racial and ethnic categories
[4]Excluded subjects with MI
[5]Values are mean ± SD
[6]Values are median
[7]n=19, excluded a subject who had his first visit at 4.98 months of age
[8]Number of upper respiratory tract infections requiring antibiotics in the first 2 years of life
[9]Number of hospitalizations in the first 2 years of life, excluding hospitalization for MI
[10]Number of *Pseudomonas aeruginosa* acquired in the first 2 years of life

Table 2. Selected fatty acid concentrations (expressed as weight % of total identified fatty acids) at each time point

	4M (n=93)		12M (n=93)		24M (n=104)		p
	Mean ± SD	**Median (IQR)[1]**	**Mean ± SD**	**Median (IQR)[1]**	**Mean ± SD**	**Median (IQR)[1]**	
SFA							
Myristic, 14:0	0.46 ± 0.15	0.42 (0.34-0.56)	0.44 ± 0.14	0.43 (0.32-0.52)	0.40 ± 0.12	0.39 (0.32-0.47)	0.005
Palmitic, 16:0	23.02 ± 1.25	23.12 (22.21-23.73)	23.55 ± 1.03	23.70 (23.09-24.21)	23.48 ± 1.12	23.57 (22.68-24.09)	0.003
Stearic, 18:0	19.29 ± 1.02	19.28 (18.71-19.90)	18.57 ± 0.77	18.60 (18.17-18.98)	18.93 ± 0.87	18.99 (18.33-19.50)	<0.001
Other[2]	4.30 ± 1.76	5.24 (2.39-5.91)	5.11 ± 1.71	5.88 (3.29-6.35)	4.70 ± 1.79	5.49 (2.94-6.08)	0.003
MUFA							
Palmitoleic, 16:1n-7	0.22 ± 0.11	0.21 (0.15-0.26)	0.22 ± 0.10	0.20 (0.14-0.27)	0.30 ± 0.16	0.28 (0.20-0.36)	<0.001
Oleic, 18:1n-9	13.69 ± 1.63	13.37 (12.65-14.75)	14.26 ± 1.32	14.19 (13.27-15.16)	14.74 ± 1.63	14.51 (13.64-15.94)	<0.001
Other[3]	1.69 ± 0.31	1.66 (1.46-1.86)	1.50 ± 0.23	1.48 (1.36-1.60)	1.65 ± 0.23	1.64 (1.50-1.79)	<0.001
PUFA							
LA, 18:2n-6	10.32 ± 1.32	10.39 (9.39-11.19)	11.00 ± 1.35	10.91 (9.89-12.03)	11.15 ± 1.52	11.16 (10.04-12.17)	<0.001
GLA, 18:3n-6	0.06 ± 0.02	0.05 (0.04-0.07)	0.06 ± 0.02	0.06 (0.04-0.07)	0.09 ± 0.03	0.08 (0.07-0.10)	<0.001
DGLA, 20:3n-6	1.93 ± 0.67	1.84 (1.49-2.11)	1.72 ± 0.43	1.70 (1.34-1.97)	2.02 ± 0.41	1.97 (1.71-2.26)	<0.001

AA, 20:4n-6	18.63 ± 1.46	18.45 (17.50-19.31)	17.72 ± 1.58	17.84 (16.75-18.53)	17.60 ± 1.97	17.63 (16.06-18.60)	<0.001
ALA, 18:3n-3	0.21 ± 0.21	0.17 (0.13-0.20)	0.21 ± 0.12	0.19 (0.15-0.24)	0.22 ± 0.10	0.21 (0.17-0.25)	0.860
EPA, 20:5n-3	0.24 ± 0.13	0.20 (0.16-0.30)	0.52 ± 1.03	0.28 (0.19-0.43)	0.72 ± 1.21	0.46 (0.37-0.63)	0.002
DHA, 24:6n-3	5.67 ± 1.16	5.51 (4.97-6.55)	4.93 ± 1.15	4.88 (4.15-5.94)	3.78 ± 1.33	3.49 (2.82-4.66)	<0.001
Mead acid, 20:3n-9	0.27 ± 0.64	0.09 (0.06-0.30)	0.19 ± 0.34	0.07 (0.04-0.28)	0.23 ± 0.27	0.18 (0.09-0.31)	0.004
Ratios							
Triene:tetraene	0.02 ± 0.04	0.01 (0.00-0.02)	0.01 ± 0.02	0.00 (0.00-0.02)	0.01 ± 0.02	0.01 (0.01-0.02)	0.617
AA/DHA	3.41 ± 0.68	3.28 (2.95-3.89)	3.81 ± 0.99	3.59 (2.95-4.30)	5.30 ± 2.02	5.14 (3.59-6.63)	<0.001
AA/LA	1.83 ± 0.27	1.80 (1.61-2.05)	1.63 ± 0.23	1.64 (1.48-1.81)	1.60 ± 0.26	1.59 (1.43-1.76)	<0.001
LA/DGLA	5.86 ± 1.86	5.70 (4.68-6.86)	6.82 ± 1.88	6.31 (5.54-8.06)	5.74 ± 1.41	5.77 (4.60-6.57)	<0.001

[1]IQR: Interquartile range
[2]Other saturated fatty acids include caprylic acid (8:0), capric acid (10:0), lauric acid (12:0), arachidic acid (20:0), and lignoceric acid (24:0).
[3]Other monounsaturated fatty acids include sapienic acid (16:1n-10), vaccenic acid (18:1n-7), and 11-eicosenoic acid (20:1n-9).

Table 3. Dietary intake per day by EFA status at 24M[1]

	EFAS (n=26)		EFAI (n=23)		EFAD (n=6)		p
	Mean ± SD	**Median (IQR)[2]**	**Mean ± SD**	**Median (IQR)[2]**	**Mean ± SD**	**Median (IQR)[2]**	
EER[3]	1078 ± 372	1010 (939-1095)	1030 ± 108	1050 (952-1117)	990 ± 76	992 (935-1046)	0.721
Energy intake, kcal/day	1322 ± 354	1294 (1153-1396)	1378 ± 448	1339 (1075-1719)	1281 ± 196	1310 (1077-1360)	0.676
% EER	129 ± 37	130 (106-148)	136 ± 50	134 (95-164)	130 ± 23	129 (119-136)	0.749
CHO, g/day	158.0 ± 46.1	147.8 (125.0-185.7)	161.7 ± 43.2	172.4 (122.6-189.6)	152.3 ± 13.6	147.9 (146.7-161.0)	0.827
% kcal from CHO	48.1 ± 8.3	48.4 (45.2-52.7)	48.7 ± 9.4	49.4 (43.9-56.7)	48.3 ± 6.4	49.4 (45.9-51.4)	0.977
Protein, g/day	42.2 ± 11.6	41.5 (36.1-47.9)	49.0 ± 18.0	50.2 (35.3-63.1)	44.6 ± 9.5	45.6 (36.7-54.1)	0.172
% kcal from protein	12.8 ± 2.2	12.4 (11.1-14.5)	14.2 ± 3.0	14.1 (11.8-16.1)	13.9 ± 1.4	13.7 (13.4-14.1)	0.058[4]
Total fat, g/day	60.1 ± 23.4	56.0 (45.0-64.8)	61.7 ± 33.5	53.8 (38.7-78.6)	56.0 ± 16.8	53.8 (44.1-58.9)	0.760
% kcal from fat	40.5 ± 8.3	39.3 (35.0-44.0)	38.4 ± 8.9	37.8 (31.0-42.7)	38.7 ± 5.8	36.4 (36.0-41.0)	0.569
SFA, g/day	24.08 ± 11.52	22.60 (16.72-26.74)	27.16 ± 19.47	19.54 (16.57-30.51)	22.50 ± 8.27	21.42 (18.87-24.26)	0.605
MUFA, g/day	19.29 ± 9.07	16.91 (13.65-21.81)	18.45 ± 9.91	16.72 (10.49-23.89)	16.58 ± 9.06	13.70 (11.64-17.96)	0.707
PUFA, g/day	9.29 ± 4.87	8.45 (6.30-10.89)	9.45 ± 4.66	8.47 (5.26-13.79)	10.85 ± 6.14	9.37 (6.45-11.61)	0.789
Omega 6, g/day	7.59 ± 3.93	6.95 (4.63-9.57)	7.20 ± 3.87	6.41 (3.96-10.58)	6.72 ± 7.06	4.74 (3.33-6.74)	0.906
Omega 3, g/day	0.98 ± 0.52	0.88 (0.61-1.10)	1.07 ± 0.64	0.96 (0.58-1.53)	0.84 ± 0.58	0.88 (0.45-0.97)	0.592

Omega6:Omega3	8.43 ± 3.12	8.30 (6.73-10.22)	7.54 ± 3.58	6.18 (5.21-9.14)	7.18 ± 2.93	6.93 (5.17-9.11)	0.604
Selected fatty acids							
LA, g/day	7.54 ± 3.92	6.89 (4.62-9.54)	7.13 ± 3.82	6.38 (3.94-10.10)	7.87 ± 6.30	6.04 (4.09-7.40)	0.975
% kcal from LA	5.01 ± 1.71	5.06 (3.65-6.84)	4.43 ± 1.50	4.22 (3.21-5.78)	5.32 ± 3.36	4.81 (2.71-5.75)	0.531
ALA, g/day	0.95 ± 0.52	0.87 (0.59-1.07)	1.01 ± 0.58	0.95 (0.57-1.40)	0.97 ± 0.43	0.92 (0.80-0.96)	0.892
% kcal from ALA	0.64 ± 0.29	0.63 (0.48-0.74)	0.64 ± 0.26	0.61 (0.40-0.79)	0.68 ± 0.23	0.66 (0.63-0.80)	0.949
AA, g/day	0.05 ± 0.06	0.03 (0.02-0.07)	0.07 ± 0.10	0.03 (0.01-0.09)	0.03 ± 0.04	0.01 (0.01-0.03)	0.613
DHA, g/day	0.01 ± 0.03	0.00 (0.00-0.01)	0.03 ± 0.10	0.00 (0.00-0.01)	0.00 ± 0.01	0.00 (0.00-0.00)	0.618

[1]Phenotype was included as a covariate and was not significant for all intake variables
[2]IQR: Interquartile range
[3]EER: Estimated Energy Requirement. EER for 1-3 years of age was calculated based on the formula (89 x weight (kg) – 100) + 20
[4]Phenotype as a covariate was significant in the model, p=0.046; subjects who were PS consumed significantly higher protein (expressed as % kcal) than subjects who had MI

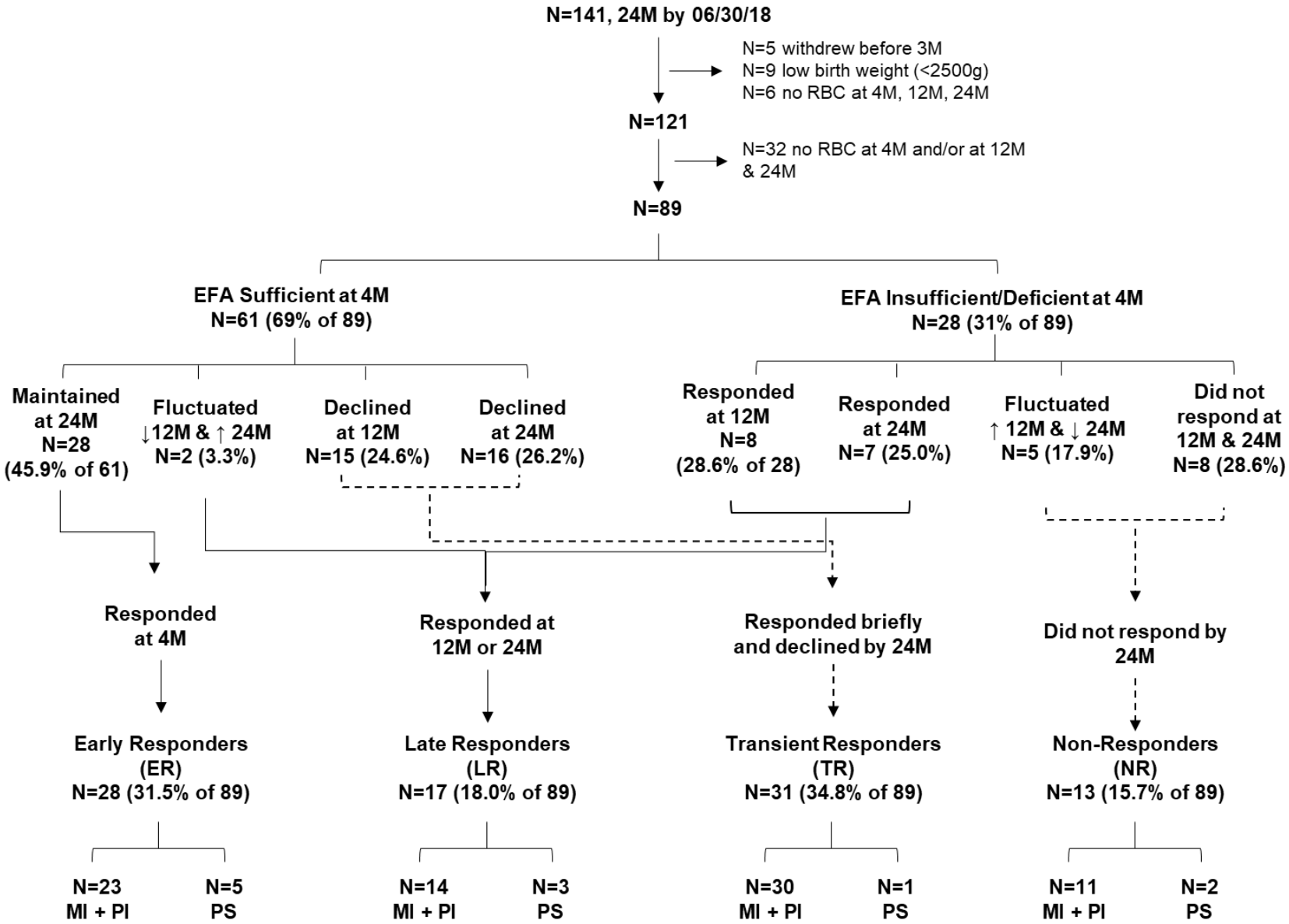

Figure 1. Study population and classification of essential fatty acid (EFA) responder status. N=89 included subjects who were PS and excluded subjects who did not have a baseline EFA measured in red blood cells

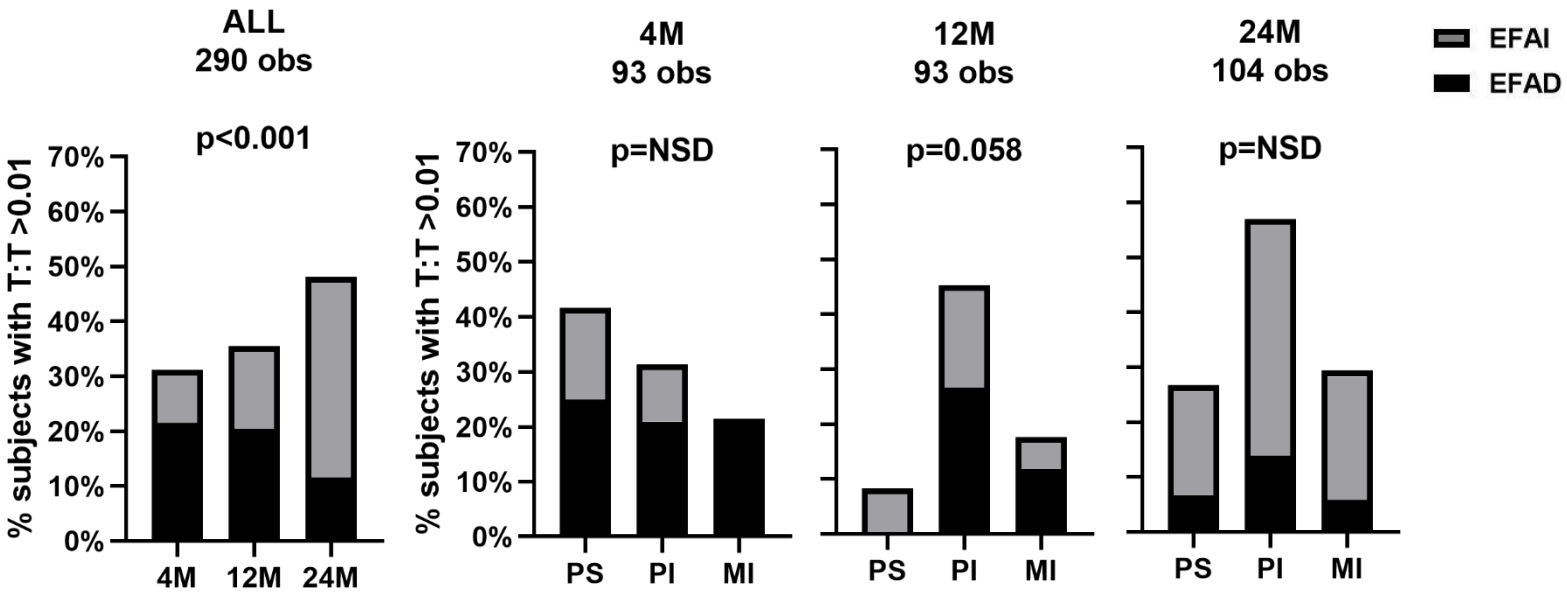

Figure 2. Prevalence of EFAD and EFAI in our cohort in the first 2 years of life

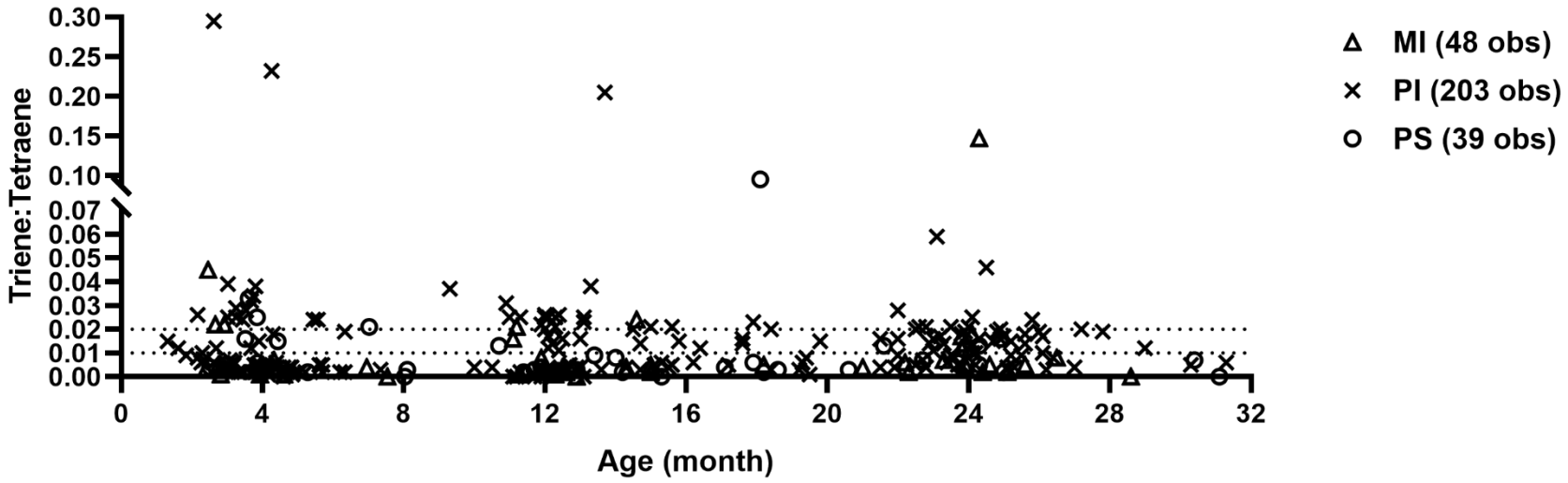

Figure 3. Scatterplot of triene:tetraene with age

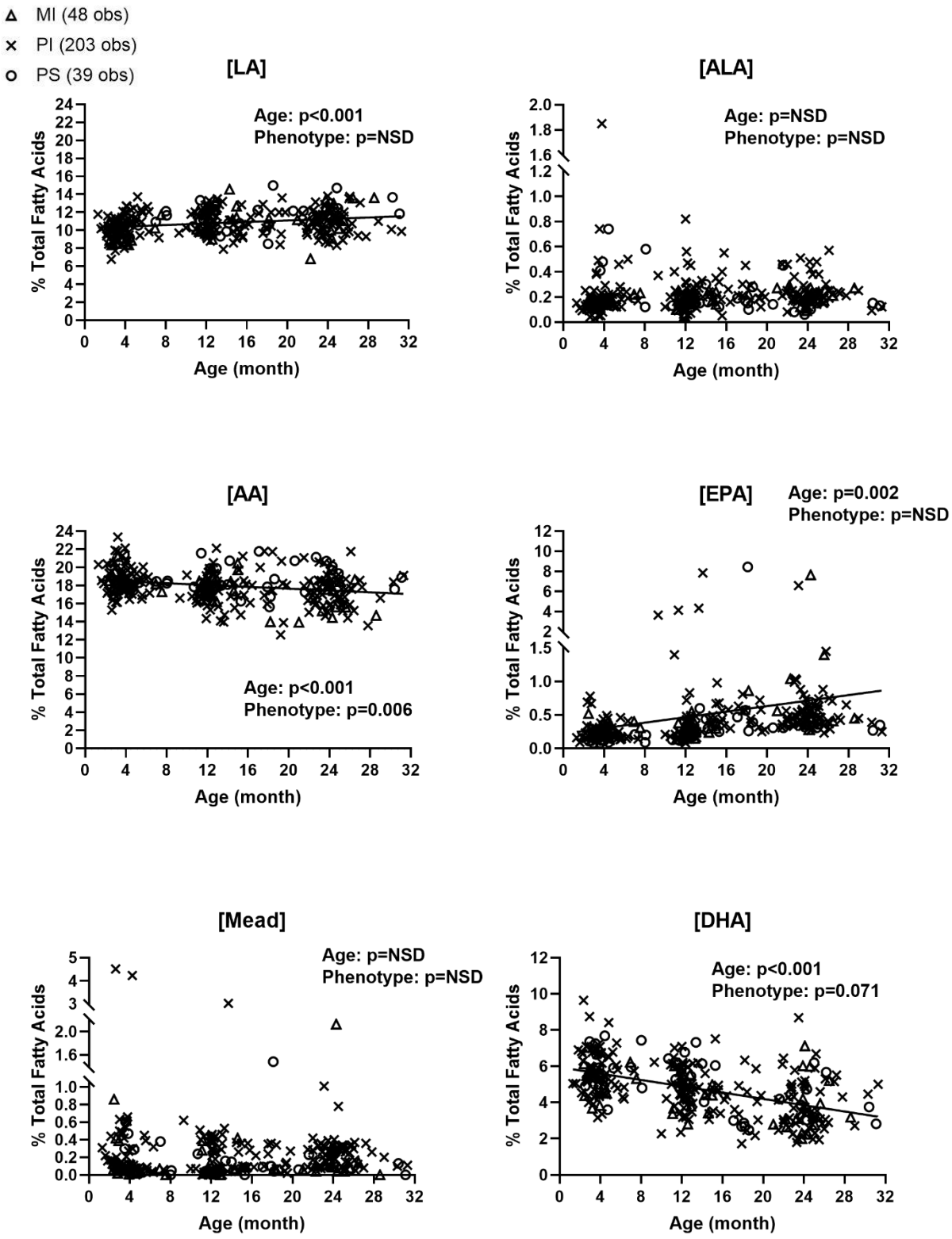

Figure 4. Age-related changes for selected fatty acids

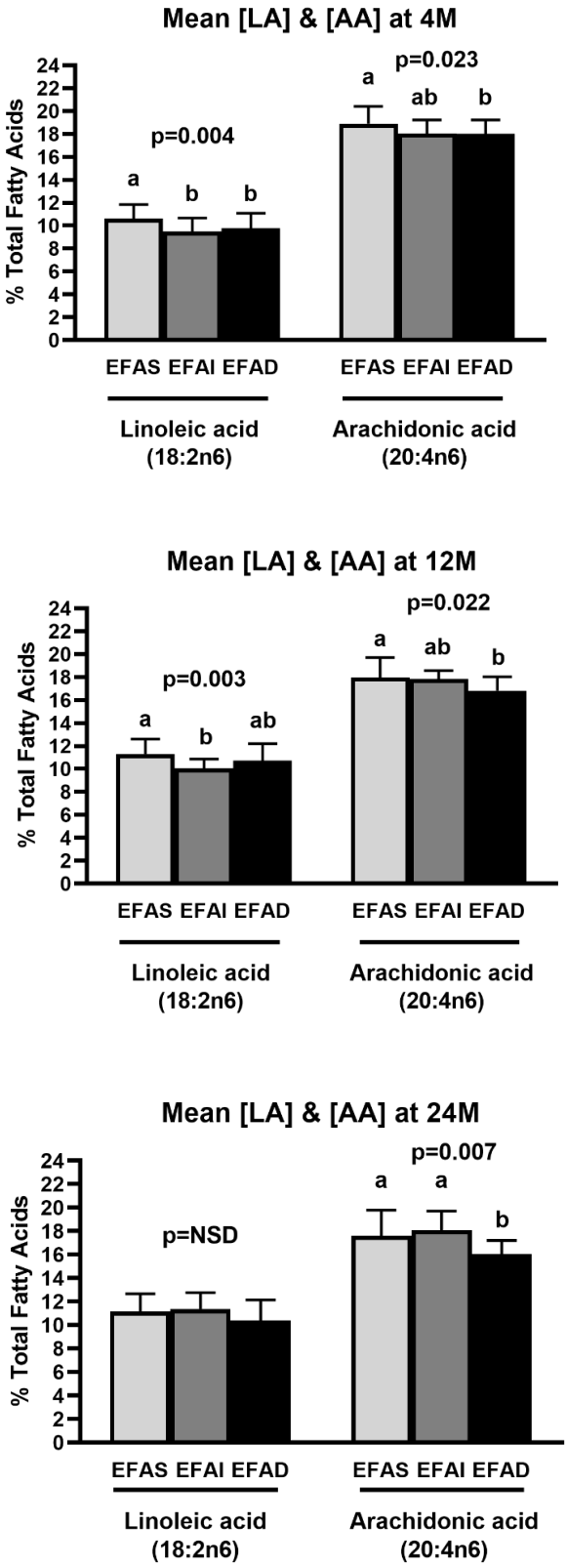

Figure 5. Mean LA and AA concentrations among EFA status at 4M, 12M, and 24M

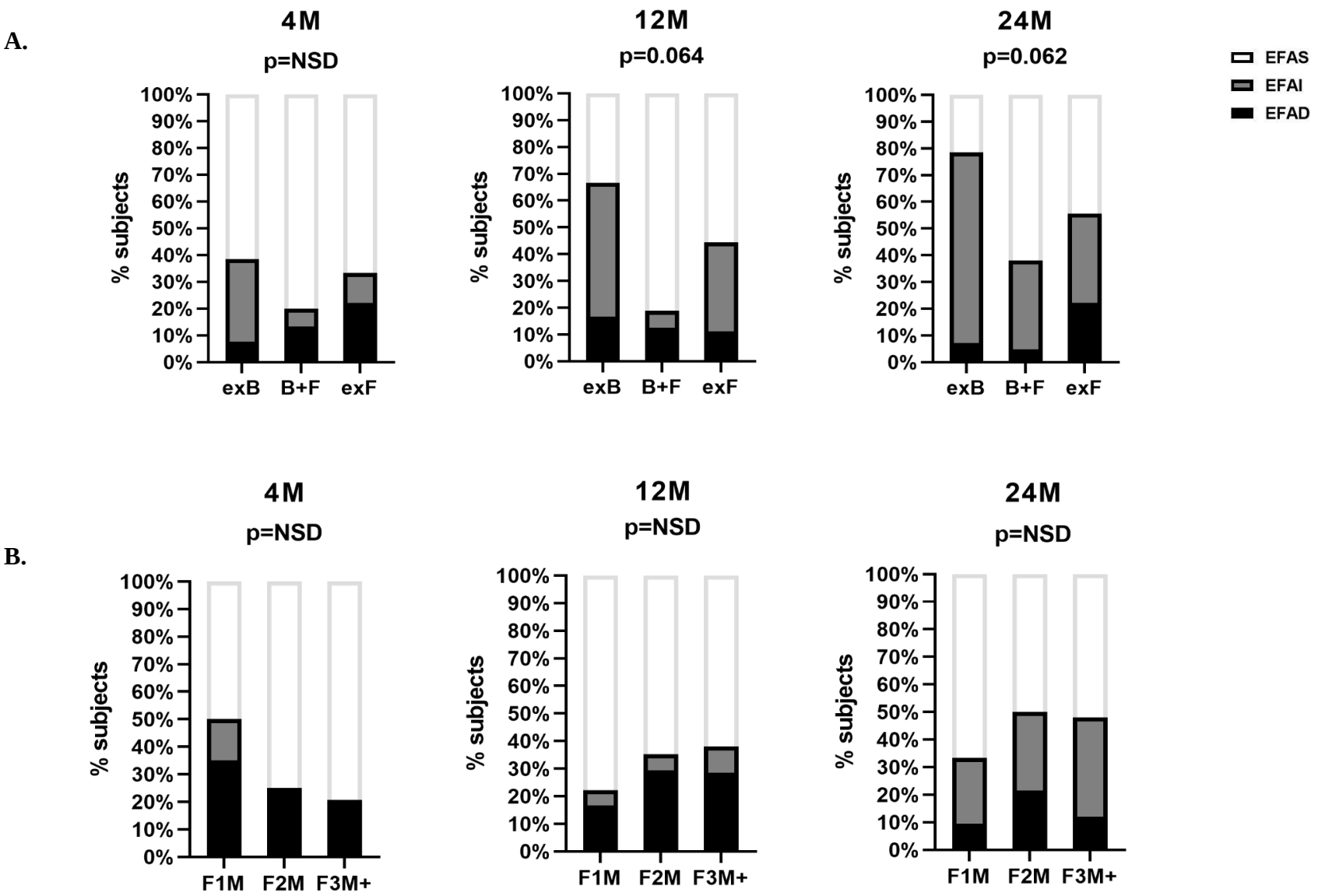

Figure 6. EFA status distribution among (A) subjects on unfortified feedings (exclusive breastfeeding, breastfeeding and formula, and exclusive formula) and (B) fortified feedings (fortification at 1 month, 2 months, and 3 months and beyond)

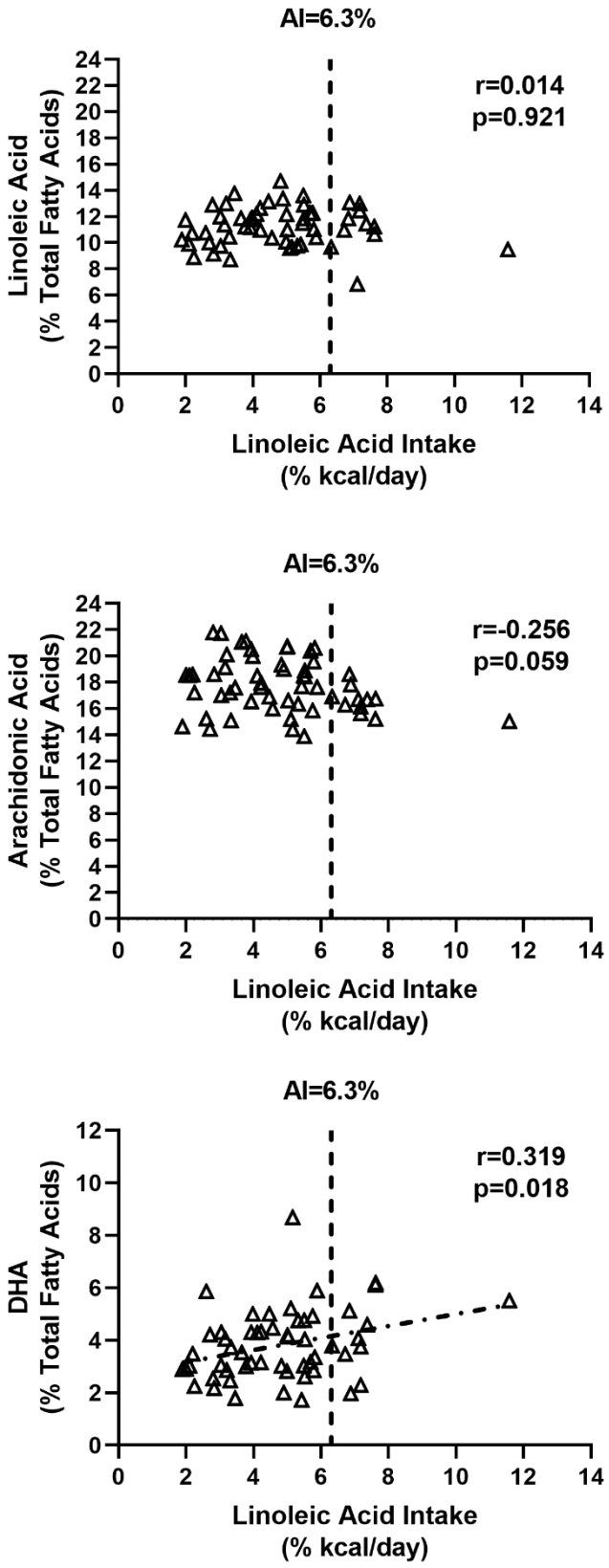

Figure 7. Correlations between LA, AA, and DHA and LA dietary intake (% kcal/day) at 24M

CHAPTER 5

INFLAMMATORY MARKERS IN YOUNG CHILDREN WITH CYSTIC FIBROSIS IN THE FIRST 2 YEARS OF LIFE AND THEIR ASSOCIATIONS WITH ESSENTIAL FATTY ACIDS

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Abstract

Background: Cystic fibrosis (CF) is a progressive disease and is characterized by neutrophil-dominated infiltration of the airways and recurrent lung infections. The inflammatory response in CF is a balance of pro-inflammatory and anti-inflammatory mediators.

Objectives: To describe some of the commonly reported cytokines in CF in the first 2 years of life and explore the associations between fatty acids and inflammatory markers.

Design: Data from an ongoing multicenter prospective longitudinal observational study at 5 CF centers in the US were analyzed. Fatty acids in red blood cells, plasma C-reactive protein, and plasma cytokines (IL-1β, IL-6, IL-8, IL-10, and TNF-α) were quantified at 3 time points in the 2 years.

Results: About 30% of specimens had undetectable CRP concentrations and 40% had undetectable IL-6 concentrations. Only 5% of our subjects had elevated CRP concentrations. IL-6 increased significantly with age in the first 2 years of life and subjects who were pancreatic insufficient had significantly higher IL-6 and IL-1β concentrations but lower CRP concentrations than subjects who had meconium ileus. CRP was not correlated with IL-1β, IL-6, IL-8, IL-10, and TNF-α, and were higher in subjects who were essential fatty acid deficient. IL-8 and TNF-α were negatively correlated with eicosanoid precursor, arachidonic acid, and IL-8 was also negatively correlated with linoleic acid.

Conclusion: Our findings are of clinical relevance and disagree with findings in the literature regarding LA supplementation and its pro-inflammatory effects. Our study provided evidence for future supplementation trials to generate new evidence for supplementation in clinical practice guidelines.

Keywords: cystic fibrosis, cytokines, inflammatory markers, CRP, essential fatty acids, infants

INTRODUCTION

Cystic fibrosis (CF) is a progressive disease and is characterized by neutrophil-dominated infiltration of the airways and recurrent lung infections (1). Even in asymptomatic infants, there is evidence that airway infection and inflammation are present in infants as young as 3 months of age (2-4). Inflammation is closely associated with chronic infections and is critical in disease progression in CF. The inflammatory response in CF is usually characterized by high pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α concentrations and low anti-inflammatory cytokines such as IL-10 concentrations (1, 5).

Studies have found that in the bronchoalveolar lavage fluid (BAL) of infants and young children with CF, an increase in inflammatory markers such as IL-1β, IL-8, and TNF-α were observed, indicating the presence of inflammation in the airways (2, 3, 5). Moreover, other studies have shown that TNF-α, IL-1β, and IL-8 concentrations were increased in the CF population compared to healthy controls and during pulmonary exacerbations compared to when clinically stable (2, 5, 6). Bonfield et al. were the first to show that IL-10 concentrations in BAL were lower in CF patients than in controls (5).

In addition to airway infections, it has been proposed that abnormal essential fatty acid (EFA) metabolism could be related to inflammation in CF (7, 8). It has been hypothesized that free arachidonic acid (AA, 20:4n-6) released from cell membrane phospholipids as a result of increased phospholipase A_2 activity in CF could potentially increase cytokine response (9). AA is the dominant substrate used for the synthesis of eicosanoids such as prostaglandins and leukotrienes. Eicosanoids are involved in modulating the intensity and duration of inflammatory responses; AA can be oxidized to produce leukotrienes which in turn generates a pro-inflammatory cascade (10, 11).

Multiple studies have investigated fatty acids and inflammatory responses in the CF population separately, but few have looked at their relationships in tandem and most described cross-sectional data and were usually during times of pulmonary exacerbations (12-16). One study investigated the correlations between clinical parameters and inflammatory markers in sputum and serum; the authors found that IL-8 and TNF-α were negatively correlated with lung function and there were no significant association between cytokines in sputum and serum (12). Another study found that supplementation with a small dose of n-6 and n-3 fatty acids for a year lowered TNF-α in blood and is associated with reduced number of pulmonary exacerbations (13).

Our study is one of the few in the recent years to explore cytokine concentrations in infants from various CF centers in a longitudinal fashion. The objectives of this paper are to describe some of the commonly reported cytokines in CF in the first 2 years of life and explore the associations between fatty acids and inflammatory markers in clinically stable children. Since our study is an observational study, our findings regarding the cytokine profile in infants with CF reflect current clinical practice and the association between fatty acids and inflammatory markers in infants will add new evidence to the field.

SUBJECTS AND METHODS

Study design and population

The FIRST (*Feeding Infants Right… from the STart*) study is a multicenter, prospective, observational study conducted at 5 CF centers with the aim to identify optimal feeding for infants with CF. Details of the study have been described in the previous chapter (see Chapter 4). Of the 178 subjects enrolled in the study, 141 subjects were at least 24 months of age by 06/30/2018. Five infants withdrew before 3 months of age, 9 had a low birth weight (<2500 g), and another 6 did not have red blood cell (RBC) specimens in the first 2 years of life; the final analyses were done on 121 subjects.

Classification of gastrointestinal phenotype and pancreatic status

The most severe gastrointestinal phenotype in infants with CF is the neonatal presentation of meconium ileus (MI), which occurs in approximately 20% of infants with CF (17, 18). In the FIRST study, fecal specimens were collected at enrollment (approximately 2 months), 4, 6, 8, 12, and 24 months of age. All infants born with MI were also pancreatic insufficient (PI). Infants were classified as pancreatic sufficient (PS) if their fecal elastase-1 concentrations were consistently >200 μg/g in their first 2 years of life. Therefore, we classified our study population into 3 gastrointestinal phenotypes – MI, PI, and PS.

Laboratory analyses

Blood specimens were obtained at approximately 4 months (4M), 12 months (12M), and 18 to 24 months (24M) of age. Fatty acids were measured in RBC by gas liquid chromatography (Agilent 6890 Series, Santa Clara, CA) with a flame ionization detector. We modified a

simplified method for quantitative analysis of fatty acids with the addition of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) as internal controls (19). Boron trifluoride (BF_3) in 14% methanol (Sigma-Aldrich, St. Louis, MO) was added directly to the homogenized samples for methylation, followed by extraction with hexane. Chromatograms were analyzed using HP ChemStation Software. Results were normalized to C15:0 and each fatty acid was expressed as a weight percent of total identified fatty acids in RBC. A total of 290 RBC specimens from 121 subjects were analyzed for fatty acids.

The MILLIPLEX® MAP human cytokine/chemokine magnetic bead panel assay (Millipore, Billerica, MA) was used to quantify plasma IL-1β, TNF-α, IL-6, IL-8, and IL-10 according to manufacturer's instructions. Plasma specimens were also sent to Clinical Laboratory Services of the University of Wisconsin Hospitals and Clinics for measurement of high-sensitivity C-reactive protein (CRP). A total of 257 plasma specimens were analyzed for cytokines and 242 plasma specimens were analyzed for CRP.

Classification of essential fatty acid status and elevated CRP

Triene (mead acid)-to-tetraene (arachidonic acid) ratio (T:T) was used to define EFA status from RBC specimens since they represent long term (20). EFA deficiency (EFAD) was defined as T:T >0.02, EFA insufficiency (EFAI) was defined as 0.01< T:T ≤0.02, and EFA sufficiency (EFAS) was defined as T:T ≤0.01 (21). CRP >5mg/L is suggestive of systemic inflammation according to the reference range provided by the Clinical Laboratory Services of the University of Wisconsin Hospitals and Clinics.

Statistical analyses

Statistical analyses were performed using SAS (v9.4, SAS Institute Inc., Cary, NC); data were plotted using GraphPad Prism (v8, GraphPad Software, La Jolla, CA). Data were presented as mean ± SD unless otherwise stated; p values <0.05 were considered as significant. Cytokine concentrations were not normally distributed and were log-transformed. Cytokines and CRP that were below the limits of detection were treated as zero, however, to log transform cytokines and CRP variables, a small constant is added to the dataset. Kruskal-Wallis was used to test for differences across group comparison. Mixed-model repeated measures analysis was used to assess the association between cytokines, CRP, and selected fatty acids, including age and phenotype as covariates where applicable.

RESULTS

CRP & cytokine concentrations at each time point

The baseline characteristics of this cohort has been previously described (Chapter 4). Plasma CRP and concentrations of plasma TNF-α, IL-1β, IL-6, IL-8, and IL-10 at each time point were shown in **Table 1** and **Figure 1**. About 30% of the specimens were below the limit of detection for CRP, 41% for IL-6, and 8% for IL-1β. Plasma CRP (**Figure 1A**) and IL-8 (**Figure 1E**) concentrations were not significantly different at 4M, 12M, and 24M. IL-6 (**Figure 1B**) and IL-10 (**Figure 1C**) concentrations were significantly lower at 4M compared to 12M and 24M, p=0.002 and p=0.001 respectively. IL-1β concentrations were also significantly lower at 4M compared to 12M, p=0.014 (**Figure 1D**). TNF-α concentrations at 12M were significantly higher than at 4M and 24M, p=0.009 (**Figure 1F**).

Cytokines age-related changes and GI phenotype differences in the first 2 years of life

TNF-α, IL-8, IL-10, and CRP concentrations did not change significantly with age and phenotype in the first 2 years of life. IL-6 concentrations increased significantly with age in the first 2 years of life, p=0.017 (**Figure 2A**). Subjects who were PI had significantly higher IL-6 and IL-1β concentrations but lower CRP than subjects who had MI, p<0.001, p=0.036, and p=0.007 respectively (**Figures 2B-2D**).

Correlations between cytokine concentrations, CRP, and selected fatty acids

CRP concentrations were not significantly correlated with IL-8 (r=0.114, p=0.082), TNF-α (r=0.125,p=0.055), IL-1β (r=-0.093, p=0.168), IL-6 (r=-0.055, p=0.421), and IL-10 (r=0.116, p=0.076). We further investigated the relationship between IL-6 and CRP by classifying each

variable into tertiles, excluding specimens that were below the limits of detection and high CRP and IL-6 concentrations. High IL-6 concentrations were the top 5 percentile of all non-zero values. CRP and IL-6 concentrations were not associated with each other (**Figure 3**).

IL-8 concentrations were negatively correlated with linoleic acid (LA), AA, and DHA, p=0.042, p=0.026, and p=0.006 respectively (**Figure 4**). TNF-α was negatively correlated with AA, p<0.001 and IL-10 was negatively correlated with DHA, p=0.028, as shown in **Figure 5**.

CRP concentrations were significantly higher in subjects who were EFAD

Overall, CRP concentrations were significantly higher in subjects who were EFAD compared to subjects who were EFAI and not significantly different from those who were EFAS, p=0.027 (**Figure 6**). At baseline, CRP concentrations were significantly higher in subjects who were EFAD than subjects who were EFAS, p=0.026 and at 24M, CRP concentrations were significantly higher in subjects who were EFAD than subjects who were EFAI, p=0.017, with no significant differences in CRP concentrations among the EFA status at 12M. Using CRP >5mg/L as a cut-off for possible systemic inflammation, only 5% of our cohort had elevated CRP concentrations. Given the low prevalence of elevated CRP, there were no differences among EFA status. On the contrary, IL-1β, IL-6. IL-8, TNF-α, and IL-10 concentrations were not significantly different among EFA status.

DISCUSSION

Previous studies have shown that cytokines play an important role in the inflammatory response in CF and some evidence suggested that cytokine production could be altered in the CF population (5, 22-24). The airways of CF patients are chronically colonized with bacteria, hence the increase in inflammatory response is a concern because it contributes to the progressive nature of CF lung disease. Notably, an increase in pro-inflammatory cytokines and a decrease in anti-inflammatory cytokines were reported in patients with CF compared to otherwise healthy controls (1). As such, it is crucial to monitor cytokines in CF as a means to gauge airway inflammation.

Our study is one of its kind in recent years to explore the cytokine profile in clinically stable infants with CF in the first 2 years of life. We found that 30% of our observations had undetectable CRP and only 5% of our observations had elevated CRP. Our study also found that CRP concentrations were found to be higher in subjects who were EFA deficient, which is suggestive that higher CRP concentrations could be associated with EFA deficiency and this can be clinically relevant since EFA status is not monitored routinely.

Surprisingly, we did not find any associations between IL-6 and CRP. Both IL-6 and CRP are acute phase reactants and some studies have shown their correlations (25, 26). This discrepancy in our findings could be due to a high number of specimens that had undetectable CRP and IL-6 concentrations. Another explanation would be that IL-6 and CRP were found to be positively correlated during an acute inflammation; since the specimens collected in our study were during routine clinical visits, most of our subjects were not in an acute inflammatory state.

Another significant finding from our study is the negative association with pro-inflammatory cytokines and n-6 fatty acids. Across the wide ranges of LA (~7-15% of total fatty

acids), AA (~12-23% of total fatty acids), and DHA (~2-10% of total fatty acids), the range of cytokines remain relatively small. This indicated that changes in these fatty acids in the circulation did not contribute to huge changes in cytokine concentrations. Additionally, the negative correlations debunked the theory that higher n-6 fatty acids led to increased pro-inflammatory cytokines. Our study found that higher LA and AA concentrations were associated with lower IL-8 and TNF-α, therefore suggesting that these fatty acids are not the driver for changes in cytokine profile at least in the first 2 years of life.

Omega-6 polyunsaturated fatty acids such as LA and AA are commonly thought to be pro-inflammatory. LA is the precursor of AA in the n-6 pathway for biosynthesis of long chain fatty acids. The fatty acid composition of cell membrane phospholipids is critical in cell function and cellular responses. The critical link between fatty acids and inflammation is the precursor of eicosanoids, i.e. 20-carbon polyunsaturated fatty acids liberated from cell membrane phospholipids. In particular, elevated AA in circulation is a concern even in the general population because metabolism of AA gives rise to prostaglandins, thromboxanes, and leukotrienes, which have a number of pro-inflammatory effects (27, 28).

In CF, EFA supplementation has been an outstanding question given the prevalence of EFA abnormalities reported (29-31). According to the current CFF, there is not enough evidence to recommend for or against LA and DHA supplementation in the first 2 years of life (32). The main concern with EFA supplementation, particularly LA supplementation, in CF is that the increase in LA intake will lead to an increase in AA concentrations that could trigger a pro-inflammatory cascade.

There have been some supplementation studies in CF, but the sample sizes were usually small, the doses of the supplement were very different, and the duration of supplementation

varied significantly. A study that investigated the effects of LA supplementation in CF airways cells and $cftr^{-/-}$ transgenic mice and the authors found that addition of LA was associated with increased AA in CF cells and mice but not in wildtype cells and mice. They also reported that with LA supplementation in CF cells, an increase in IL-8 was observed and with the introduction of PGE_2, there was also an increase in IL-8 in both CF and control cells which indicated that PGE_2 stimulates IL-8 secretion (33). However, in our study, we found that IL-8 was negatively correlated with both LA and AA. One explanation for this contrast in findings could be that the addition of LA in the CF cells and $cftr^{-/-}$ mice were proportionally too high and the amounts of LA added could not be validated or translated for clinical relevance.

Furthermore, there was a study that investigated the effects of small doses of n-6 and n-3 fatty acids supplementation (including LA) for a year in patients with CF and found that AA and TNF-α concentrations were significantly lower 6 months after supplementation (13). This suggested that supplementation with LA in patients with CF does not lead to an increase in pro-inflammatory markers or in the downstream metabolite, AA, which could potentially trigger a pro-inflammatory cascade. Additionally, there was a study that looked at higher LA intake in infants with CF (12% energy vs. 7% energy) and found that infants who were on the formula with higher LA levels showed greater improvements in growth parameters (34). As an observational study, we cannot prove causation, but we have identified that higher levels of pro-inflammatory markers such as IL-8 and TNF-α were associated with lower AA concentrations.

The mixed findings in the literature suggested that even though AA is a precursor to eicosanoids, it is also a precursor to other compounds with anti-inflammatory or pro-resolving effects. In addition, the ratio of n-6 and n-3 fatty acids in circulation (35, 36). Future research on

EFA supplementation trials, incorporating LA, are warranted to monitor the effects of supplementation on inflammatory markers.

CONCLUSION

In conclusion, our study shed new light on the cytokine profile in infants with CF in the first 2 years of life and their association with fatty acids. We showed that pro-inflammatory cytokines were negatively correlated with n-6 fatty acids and that few had elevated CRP concentrations but had detectable cytokine concentrations. These findings are of clinical relevance and significance because they provided evidence for future EFA supplementation trials including LA, which was previously thought to be detrimental to the inflammatory state of CF patients. Future research on EFA supplementation and inflammatory markers are required to provide more evidence to drive current clinical practices.

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Table 1. Plasma cytokine and CRP concentrations in the first 2 years of life.

	4M			12M			24M			p[2]
	n	Median	IQR[1]	n	Median	IQR	n	Median	IQR	
IL-1β	68	2.50	1.69-4.28	80	4.26	2.20-6.27	95	3.54	2.11-5.74	0.014
TNF-α	75	26.13	21.51-35.81	85	32.05	25.30-41.30	96	26.39	20.65-35.63	0.009
IL-6	63	0.00	0.00-1.44	80	1.28	0.00-5.06	94	3.51	0.00-6.31	0.002
IL-8	75	16.18	9.71-27.13	85	17.82	12.60-27.04	95	16.58	11.99-26.15	0.532
IL-10	75	11.78	7.34-16.00	84	15.71	11.05-22.81	96	15.34	8.99-24.43	0.001
CRP	66	0.30	0.10-0.60	79	0.30	0.00-0.90	97	0.20	0.00-0.80	0.423

[1]IQR: Interquartile range
[2]Kruskal-wallis test was used

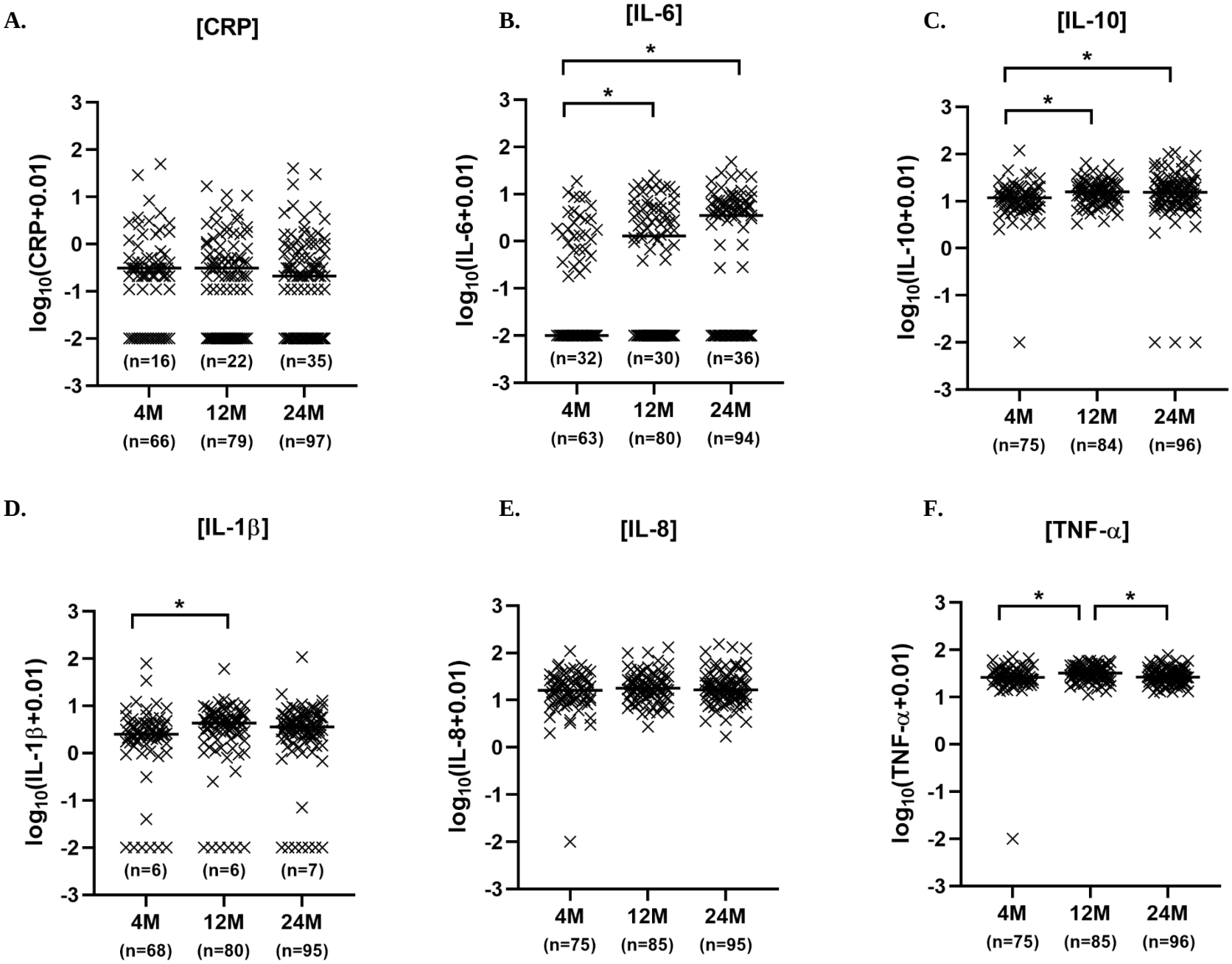

A.
[CRP]
log10(CRP+0.01)
(n=16)
(n=22)
(n=35)
4M
12M
24M
(n=66)
(n=79)
(n=97)
B.
[IL-6]
log10(IL-6+0.01)
(n=32)
(n=30)
(n=36)
(n=63)
(n=80)
(n=94)
C.
[IL-10]
log10(IL-10+0.01)
(n=75)
(n=84)
(n=96)
D.
[IL-1β]
log10(IL-1β+0.01)
(n=6)
(n=6)
(n=7)
(n=68)
(n=80)
(n=95)
E.
[IL-8]
log10(IL-8+0.01)
(n=75)
(n=85)
(n=95)
F.
[TNF-α]
log10(TNF-α+0.01)
(n=75)
(n=85)
(n=96)

Figure 1. CRP and cytokine concentrations at each time point, the horizontal line for each time point is the median. The numbers below each time point are the sample sizes and the numbers above each time point are the number of specimens which were below the limits of detection. (A) CRP concentrations did not differ significantly at each time point. (B) IL-6 concentrations were significantly lower at 4M compared to 12M and 24M, p=0.002. (C) IL-10 concentrations were significantly lower at 4M compared to 12M and 24M, p=0.001. (D) IL-1β concentrations were significantly lower at 4M compared to 12M, p=0.014. (E) IL-8 concentrations were not significantly different at each time point. (F) TNF-α concentrations at 12M were significantly higher than at 4M and 24M, p=0.009.

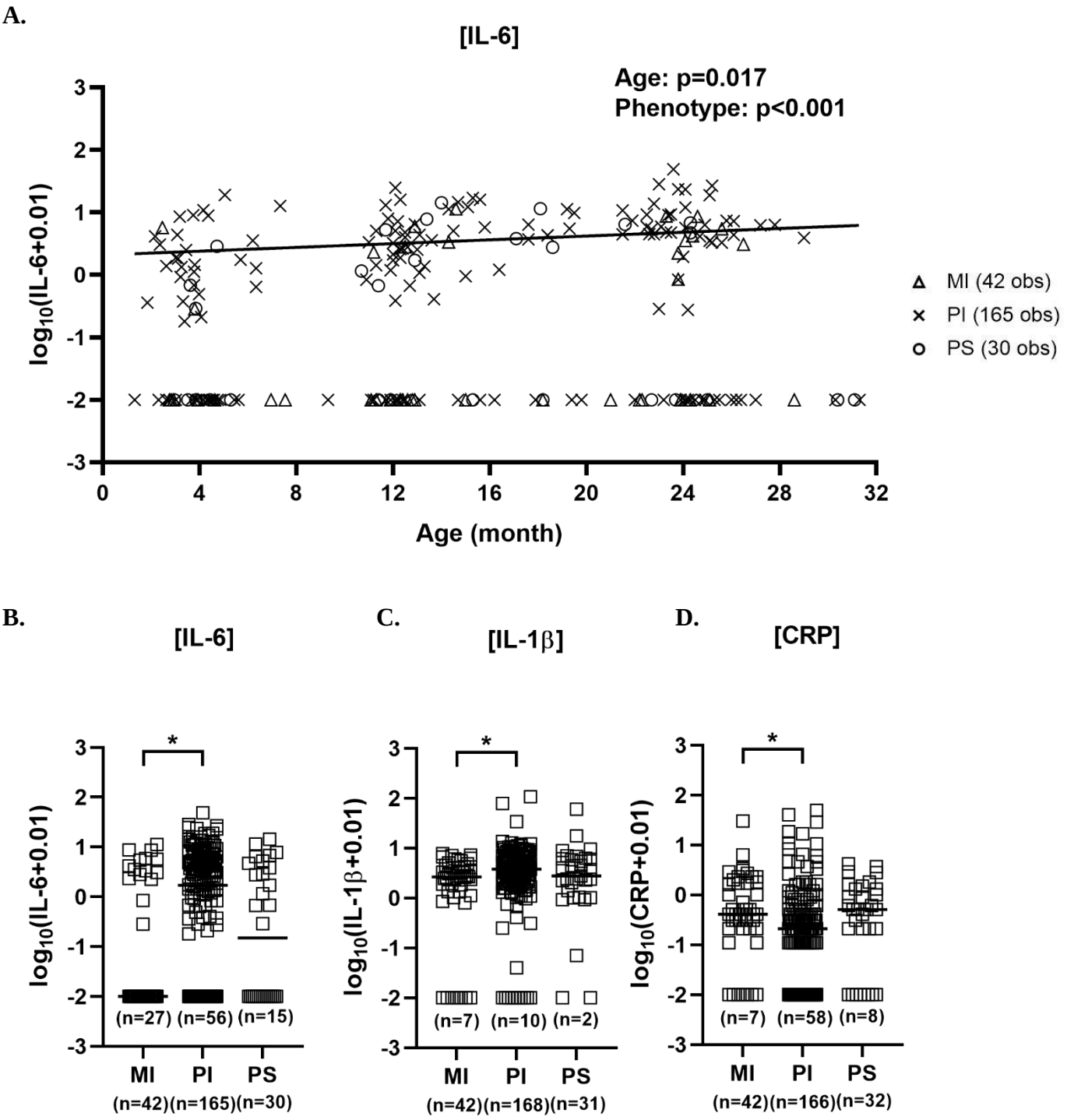
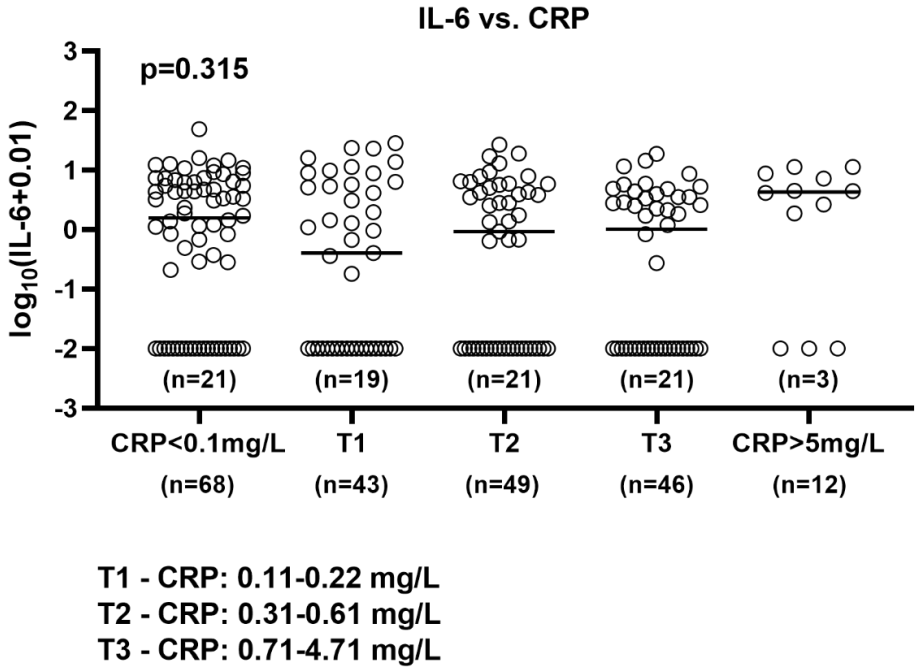


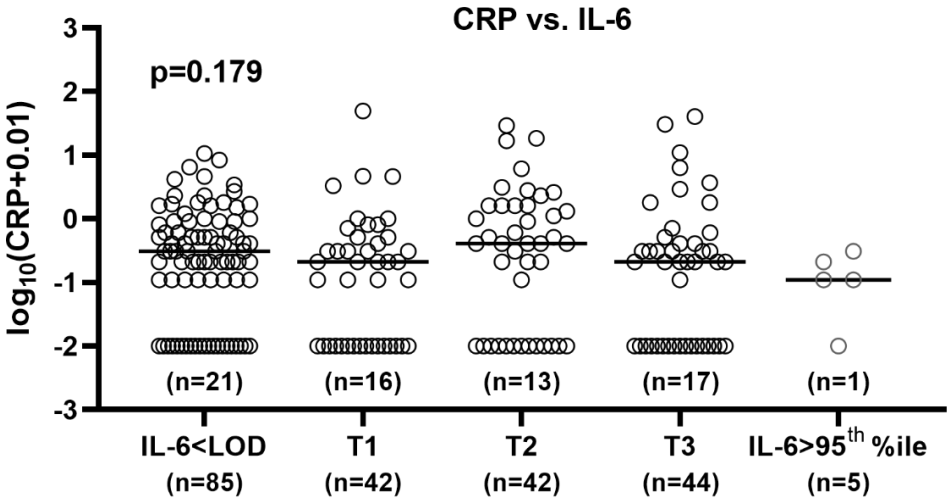
Figure 2. (A) IL-6 concentrations increased significantly with age in the first 2 years of life, p=0.017. The horizontal line across the points for each GI phenotype is the median. The sample sizes are the numbers in parentheses below the GI phenotype and the numbers above the GI phenotype are the number of specimens that were below of the limit of detection. Subjects who were PI had significantly higher (B) IL-6 and (C) IL-1β concentrations but lower (D) CRP concentrations than subjects who had MI, p<0.001, p=0.036, p=0.007 respectively.

A.



T1 - CRP: 0.11-0.22 mg/L
T2 - CRP: 0.31-0.61 mg/L
T3 - CRP: 0.71-4.71 mg/L

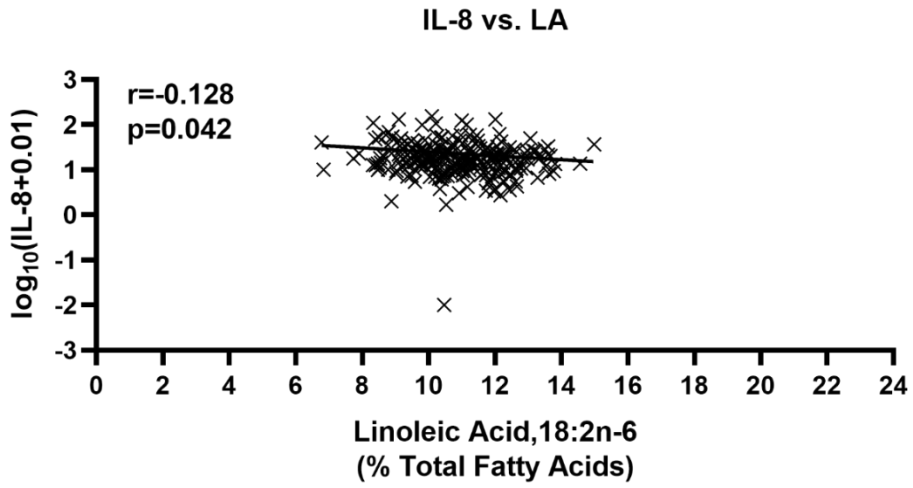
B.



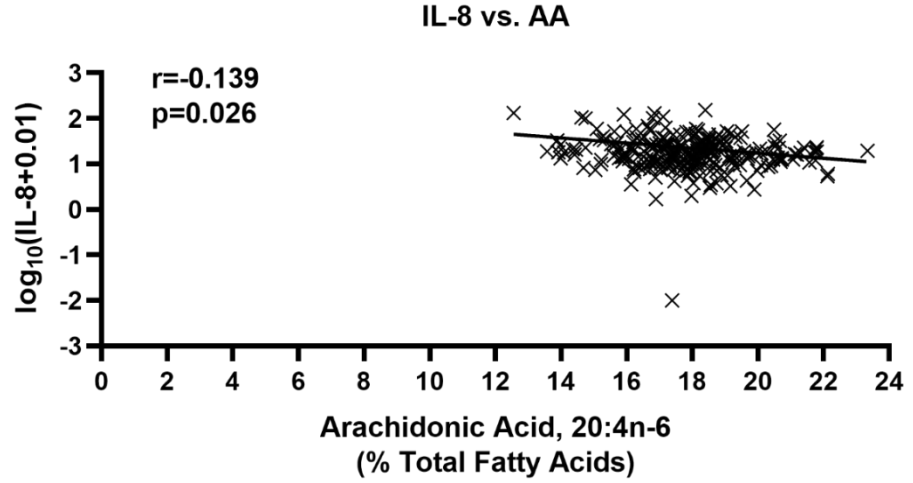
T1 - IL-6: 0.18-2.53 pg/mL
T2 - IL-6: 2.58-5.70 pg/mL
T3 - IL-6: 5.74-18.95 pg/mL

Figure 3. CRP concentrations were not associated with IL-6 concentrations. The horizontal lines represent the median. The numbers below the x-axis are the sample sizes and the numbers above are the number of specimens below the limits of detection. (A) Excluding non-zero values and high CRP (>5mg/L), CRP concentrations were divided into tertiles. (B) Excluding non-zero values and the top 5 percentile of IL-6 concentrations, the remaining IL-6 concentrations were divided into tertiles.

A.



B.



C.

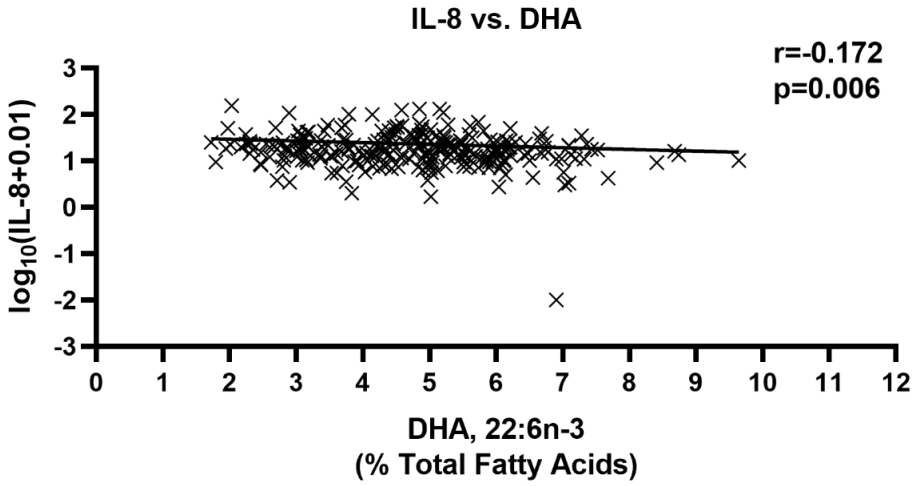
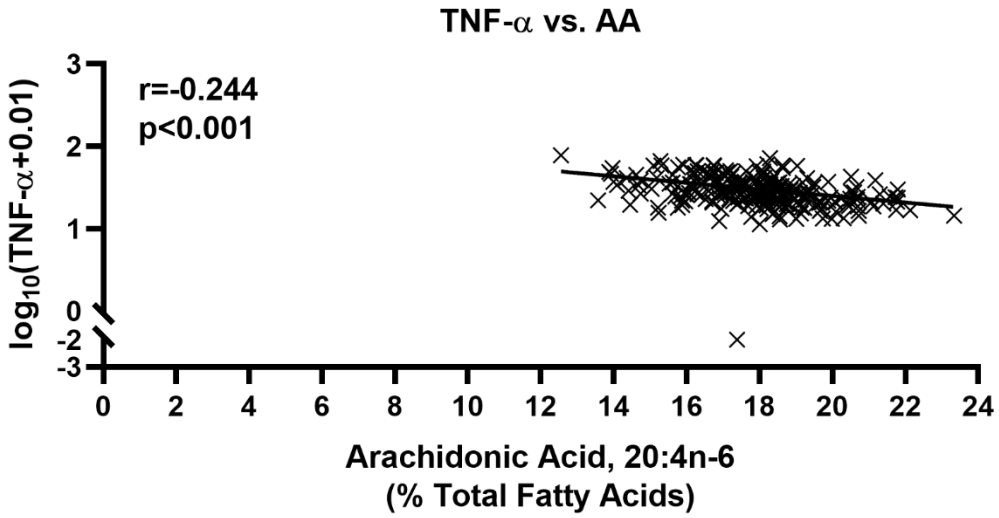


Figure 4. IL-8 concentrations were negatively correlated with (A) linoleic acid, (B) arachidonic acid, and (C) DHA.

A.



B.

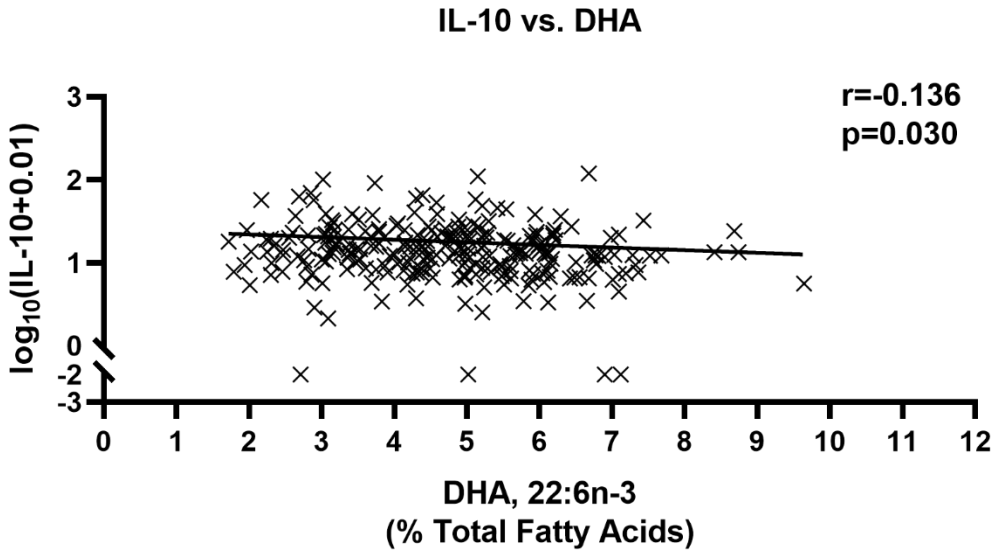


Figure 5. (A) TNF-α concentrations were negatively correlated with arachidonic acid, $p<0.001$. (B) IL-10 concentrations were negatively correlated with DHA, $p=0.030$.

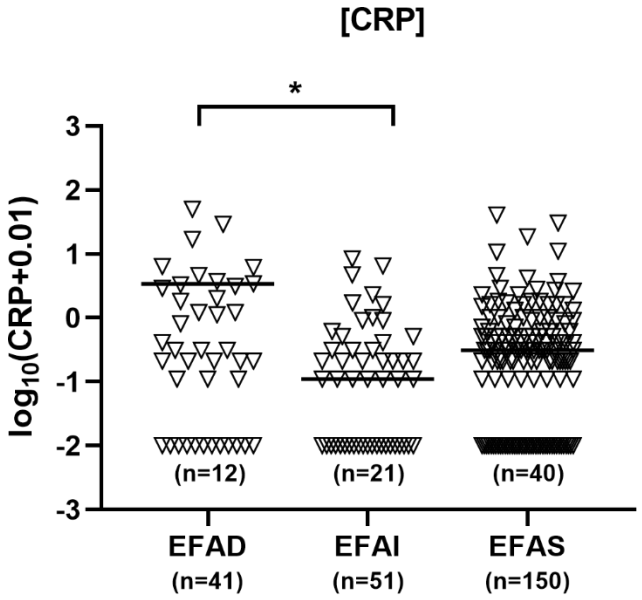


Figure 6. The horizontal lines represent the median. The numbers below the EFA status are the sample sizes and the number above are the number of specimens that were below the limit of detection. CRP concentrations were significantly higher in subjects who were EFAD compared to subjects who were EFAI and not significantly different from those who were EFAS, p=0.027.

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

This dissertation has provided the CF field with new evidence on vitamin D status, essential fatty acid status, and its association with inflammation in infants and young children with CF in the first 2 years of life.

Firstly, in Chapter 2, to answer the question whether vitamin D supplementation doses under the current clinical practice guidelines are effective in achieving optimal vitamin D status, i.e. serum 25(OH)D ≥30 ng/mL, in our cohort, we identified 4 main responses (early responder, late responder, transient responder, and non-responder) to vitamin D supplementation. Under the current recommended supplementation doses, they were effective in about 75% of our cohort (early or late responders). At 2 years of age, our cohort had significantly lower prevalence of vitamin D insufficiency compared to the otherwise healthy population, which was another indicator that the current supplementation doses were effective. However, about 10% of the CF population will be non-responders despite increasing vitamin D supplementation doses. We hypothesized that genetic variations involved in vitamin D metabolic pathways contribute to poor response to supplementation. The FIRST study is currently actively recruiting for its whole genome sequencing (WGS) study and one of its aims is to utilize data from WGS to identify genomic differences and determine genomic risk for low serum 25(OH)D concentrations.

Secondly, we reported a high prevalence (~50%) of EFA abnormalities in our cohort at 2 years of age, which prompted the suggestion that EFA status should be monitored routinely in pulmonary clinics even when the child does not have symptoms of EFA deficiency (Chapter 4). We also observed lower pro-inflammatory markers when higher n-6 fatty acids were in circulation (Chapter 5). This is promising evidence that fatty acid supplementation in CF will not trigger a pro-inflammatory cascade. Even though EFA metabolism is altered due to dysregulation of CFTR, EFA supplementation may be able to correct some of these

abnormalities. Moving forward, we would like to propose an EFA supplementation study that includes a blend of n-6 and n-3 fatty acids and explore clinical outcomes associated with supplementation. Additionally, because we know that EFA abnormalities are common in young children and EFA status is not currently monitored routinely, supplementing young children with a source of polyunsaturated fatty acids is an option. For example, Microlipid™, a fat emulsion that contains mainly safflower oil, is a good option to supplement in milk feedings or table foods to increase caloric intake as well as polyunsaturated fatty acid intake.

The FIRST study is a very comprehensive study that follows children with CF from diagnosis until 6 years of age and reflects current clinical practices. This dissertation has focused mainly in the first 2 years of life and a longitudinal analysis up to 6 years of age will be clinically relevant and significant. Moreover, with all the data collected from our cohort for multiple sub-studies, the FIRST study can evolve into a multidimensional database and advanced computational data analyses will yield more clinically meaningful results. For example, we have measured more than 38 cytokines in each plasma specimen and with the interconnectedness of the cytokine network, it will be more valuable to use cluster analysis for a more comprehensive approach to data analysis and presentation.

Similarly, since CF is a disease that involves and manifests in multiple organ systems, combining each aspect of the study is crucial. For example, we can look at our cohort holistically and identify subjects who were a non-responder in vitamin D status, EFA deficient, and had elevated CRP and cytokine concentrations. Examining the characteristics of these subjects including looking at growth parameters and genotype may shed new light on therapies because if we can predict those who will have a more severe disease state, then perhaps more aggressive and earlier therapies can be initiated before the disease progresses too rapidly.

Overall, the multifaceted nature of CF makes it complicated and patients require close monitoring of their pulmonary and nutritional status with a multidisciplinary healthcare team. The nutritional status in children with CF has improved tremendously from the past. New therapies and drugs are being developed to ease the symptoms and slow down the progressive nature of CF to preserve lung function.

APPENDIX I

ADDITIONAL DATA AND DISCUSSION TO CHAPTER 4

COMPARISON OF FATTY ACID PROFILE IN RED BLOOD CELLS AND PLASMA SPECIMENS IN INFANTS AND YOUNG CHILDREN WITH CF IN THE FIRST 2 YEARS OF LIFE

RATIONALE

Erythrocyte fatty acids may be preferred to plasma fatty acids in assessing essential fatty acid (EFA) status because red blood cell (RBC) reflect long-term fatty acid storage over the past 3 months and is less sensitive to recent intake (1). The objective of this additional data is to compare between plasma and red blood cells fatty acid profile in infants and children with CF in the first 2 years of life. We utilized the same cohort described in Chapter 4; fatty acids were measured in a total of 260 pairs of RBC and plasma specimens from the same blood draw.

RESULTS AND DISCUSSION

Fatty acid profile in plasma and RBC

The fatty acid profiles of main fatty acids were shown in **Figure 1** and stratified by each time point. Overall, the top 3 fatty acids in plasma specimens were palmitic acid, oleic acid, and LA, whereas the top 3 fatty acids in RBC specimens were palmitic acid, stearic acid, and AA. These findings were the same at each time point. Mead acid (20:3n-9) and triene:tetraene (T:T) ratio were shown in **Figure 2**. Specific fatty acids were also reported in **Table 1**.

Correlations between fatty acids in plasma and RBC

The correlation coefficients of fatty acids were illustrated in **Figure 3**. All fatty acids shown were positively correlated with each other, $p<0.001$ for all fatty acids but ALA where $p= 0.14$. Plasma and RBC mead acid, as well as plasma and RBC T:T were not correlated with each other, $r=0.007$, $p=0.907$, and $r=0.011$, $p=0.863$ respectively.

Even though low LA concentrations are commonly reported in the CF population, there is not an established cut-off for low LA concentrations in RBC and the commonly used cut-off for

low LA concentrations in plasma is 26% (2). Based on the regression equations of RBC and plasma LA concentrations, a low plasma LA of 26% corresponded to 10.5% in RBC, as shown in **Figure 4A**. At 4M, a low plasma LA of 26% is about 9.9% in RBC, this is 10.4% in RBC at 12M, and 10.9% RBC at 24M (**Figures 4B-4D**). There is no established cut-off for AA, ALA, or DHA concentrations in plasma or RBC specimens. The scatterplots of these fatty acids were shown in **Figures 5 and 6**.

In Chapter 4, we utilized T:T in RBC specimens to define EFA deficiency. However, clinically, very few clinics have the option of ordering a fatty acid panel in RBC and plasma is often used instead. The issue with plasma specimens, at least in the first 2 years of life, is that it is not possible to get non-fasting specimens and postprandial plasma specimens may result in inaccurate results. **Figure 7** showed RBC and plasma T:T. In our study, we used RBC T:T>0.02 as the cut-off for EFA deficiency (3), however, clinically, a cut-off of 0.05 is used for plasma T:T. Using both RBC and plasma T:T cut-offs, only 4% of all specimens were EFAD for both RBC and plasma, 11% were EFAD based on plasma T:T cut-off, and another 13% were EFAD based on RBC T:T cut-off. There were twice as many specimens that were EFAD in RBC specimens that were also EFAD in plasma compared to specimens that were not EFAD (24% vs. 13%), p=0.051.

Routine monitoring of EFA status is important in children with CF, yet, interpreting EFA status based on plasma T:T specimens may be tricky because of the non-fasting state of these specimens. Therefore, it is imperative to reconcile RBC and plasma cut-offs for EFA deficiency because using a RBC value or a plasma value could drive different clinical decisions.

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1. Sun Q, Ma J, Campos H, Hankinson SE, Hu FB. Comparison between plasma and erythrocyte fatty acid content as biomarkers of fatty acid intake in US women. The American Journal of Clinical Nutrition 2007;86(1):74-81.
2. Shoff SM, Ahn H-Y, Davis L, Lai H, Wisconsin CF Neonatal Screening Group. Temporal Associations Among Energy Intake, Plasma Linoleic Acid, and Growth Improvement in Response to Treatment Initiation After Diagnosis of Cystic Fibrosis. Pediatrics 2006;117(2):391-400. doi: 10.1542/peds.2004-2832.
3. Siguel EN, Chee KM, Gong JX, Schaefer EJ. Criteria for essential fatty acid deficiency in plasma as assessed by capillary column gas-liquid chromatography. Clinical Chemistry 1987;33(10):1869-73.

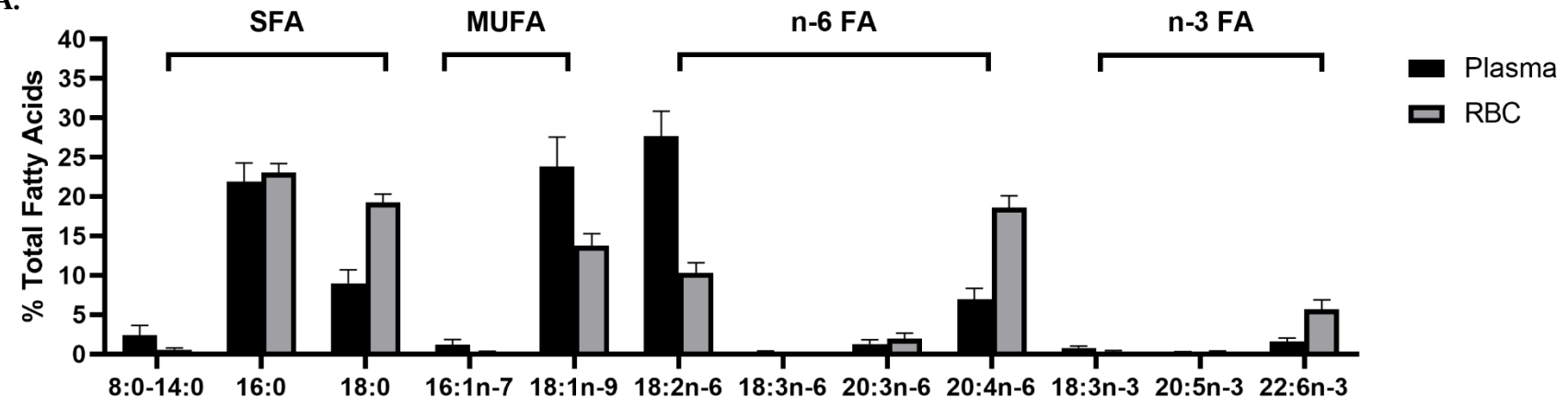
Table 1. Comparison of fatty acid concentrations in plasma and RBC at each time point

Fatty acid	4M		12M		24M	
	Plasma (n=77)	RBC (n=93)	Plasma (n=86)	RBC (n=93)	Plasma (n=97)	RBC (n=104)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
SFA						
Myristic, 14:0	1.55 ± 0.70	0.46 ± 0.15	1.58 ± 0.87	0.44 ± 0.14	1.49 ± 0.85	0.40 ± 0.12
Palmitic, 16:0	21.91 ± 2.36	23.02 ± 1.25	22.46 ± 2.27	23.55 ± 1.03	23.08 ± 2.91	23.48 ± 1.12
Stearic, 18:0	8.99 ± 1.72	19.29 ± 1.02	7.83 ± 1.11	18.57 ± 0.77	8.52 ± 1.76	18.93 ± 0.87
Other[1]	1.72 ± 0.73	4.30 ± 1.76	1.50 ± 0.69	5.11 ± 1.71	1.05 ± 0.36	4.70 ± 1.79
MUFA						
Palmitoleic, 16:1n-7	1.17 ± 0.66	0.22 ± 0.11	1.21 ± 0.58	0.22 ± 0.10	1.56 ± 0.85	0.30 ± 0.16
Oleic, 18:1n-9	23.79 ± 3.77	13.69 ± 1.63	24.50 ± 3.76	14.26 ± 1.32	24.83 ± 3.85	14.74 ± 1.63
Other[2]	1.89 ± 0.44	1.69 ± 0.31	1.90 ± 0.37	1.50 ± 0.23	2.06 ± 0.37	1.65 ± 0.23
PUFA						
LA, 18:2n-6	27.70 ± 3.14	10.32 ± 1.32	28.71 ± 3.65	11.00 ± 1.35	27.15 ± 4.90	11.15 ± 1.52
GLA, 18:3n-6	0.27 ± 0.12	0.06 ± 0.02	0.35 ± 0.15	0.06 ± 0.02	0.45 ± 0.18	0.09 ± 0.03
DGLA, 20:3n-6	1.27 ± 0.52	1.93 ± 0.67	1.25 ± 0.40	1.72 ± 0.43	1.52 ± 0.38	2.02 ± 0.41
AA, 20:4n-6	6.96 ± 1.37	18.63 ± 1.46	6.04 ± 1.38	17.72 ± 1.58	5.67 ± 1.37	17.60 ± 1.97
ALA, 18:3n-3	0.77 ± 0.25	0.21 ± 0.21	0.83 ± 0.39	0.21 ± 0.12	0.79 ± 0.42	0.22 ± 0.10
EPA, 20:5n-3	0.19 ± 0.11	0.24 ± 0.13	0.28 ± 0.18	0.52 ± 1.03	0.43 ± 0.26	0.72 ± 1.21
DHA, 24:6n-3	1.59 ± 0.44	5.67 ± 1.16	1.37 ± 0.48	4.93 ± 1.15	1.07 ± 0.50	3.78 ± 1.33

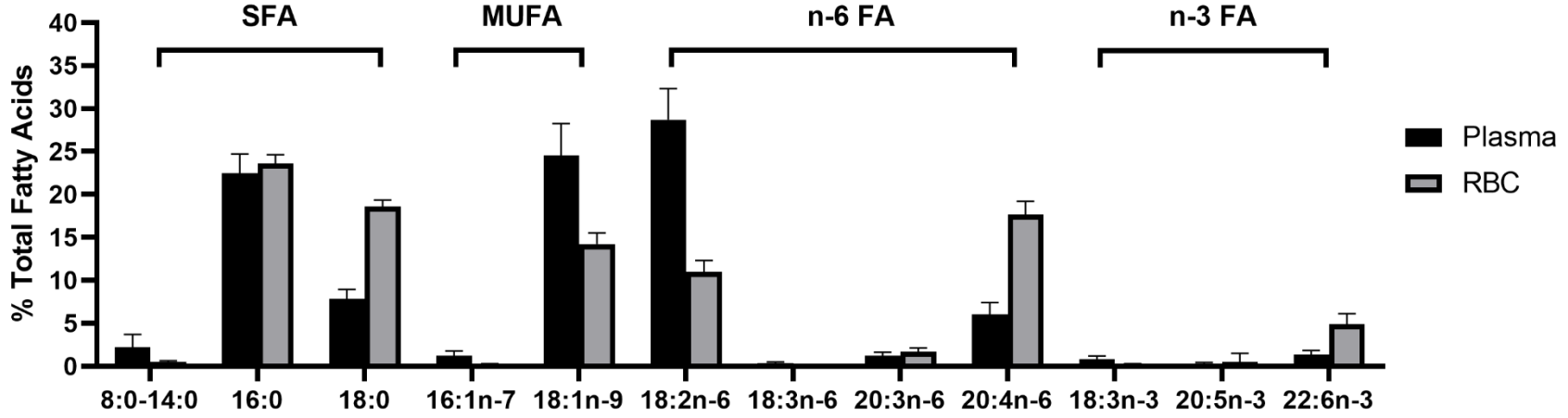
Mead acid, 20:3n-9	0.24 ± 0.59	0.27 ± 0.64	0.19 ± 0.28	0.19 ± 0.34	0.33 ± 0.90	0.23 ± 0.27

[1]Other saturated fatty acids include caprylic acid (8:0), capric acid (10:0), lauric acid (12:0), arachidic acid (20:0), and lignoceric acid (24:0).
[2]Other monounsaturated fatty acids include sapienic acid (16:1n-10), vaccenic acid (18:1n-7), and 11-eicosenoic acid (20:1n-9).

A.


SFA
MUFA
n-6 FA
n-3 FA
% Total Fatty Acids
40
35
30
25
20
15
10
5
0
8:0-14:0
16:0
18:0
16:1n-7
18:1n-9
18:2n-6
18:3n-6
20:3n-6
20:4n-6
18:3n-3
20:5n-3
22:6n-3
Plasma
RBC

B.


SFA
MUFA
n-6 FA
n-3 FA
% Total Fatty Acids
40
35
30
25
20
15
10
5
0
8:0-14:0
16:0
18:0
16:1n-7
18:1n-9
18:2n-6
18:3n-6
20:3n-6
20:4n-6
18:3n-3
20:5n-3
22:6n-3
Plasma
RBC

C.

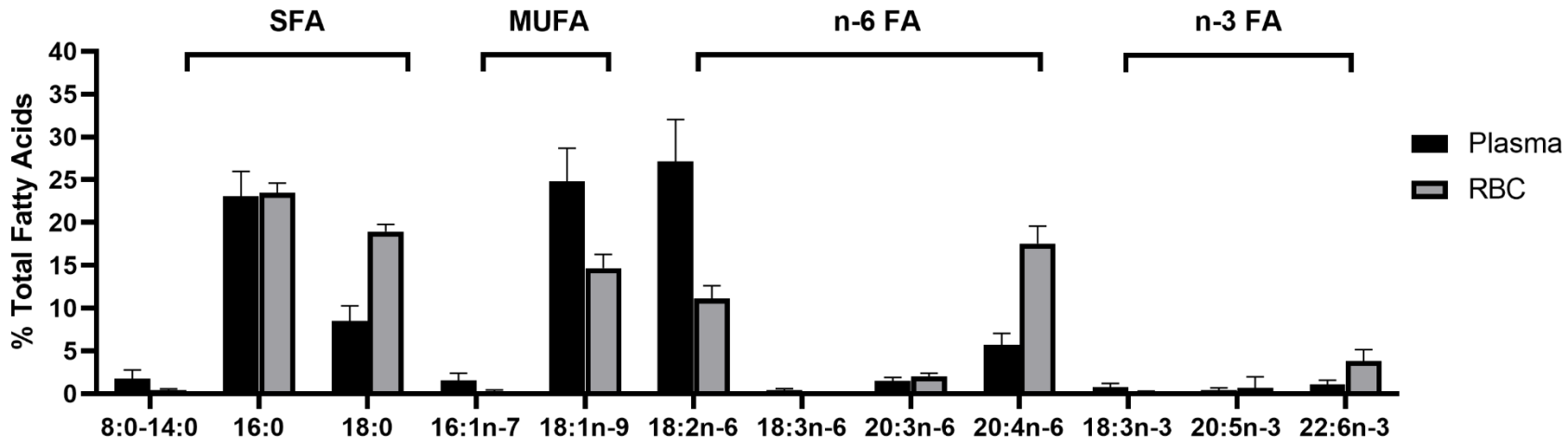


Figure 1. Selected fatty acid profile comparison at 4M (n=77), 12M (n=86), and 24M (n=97)

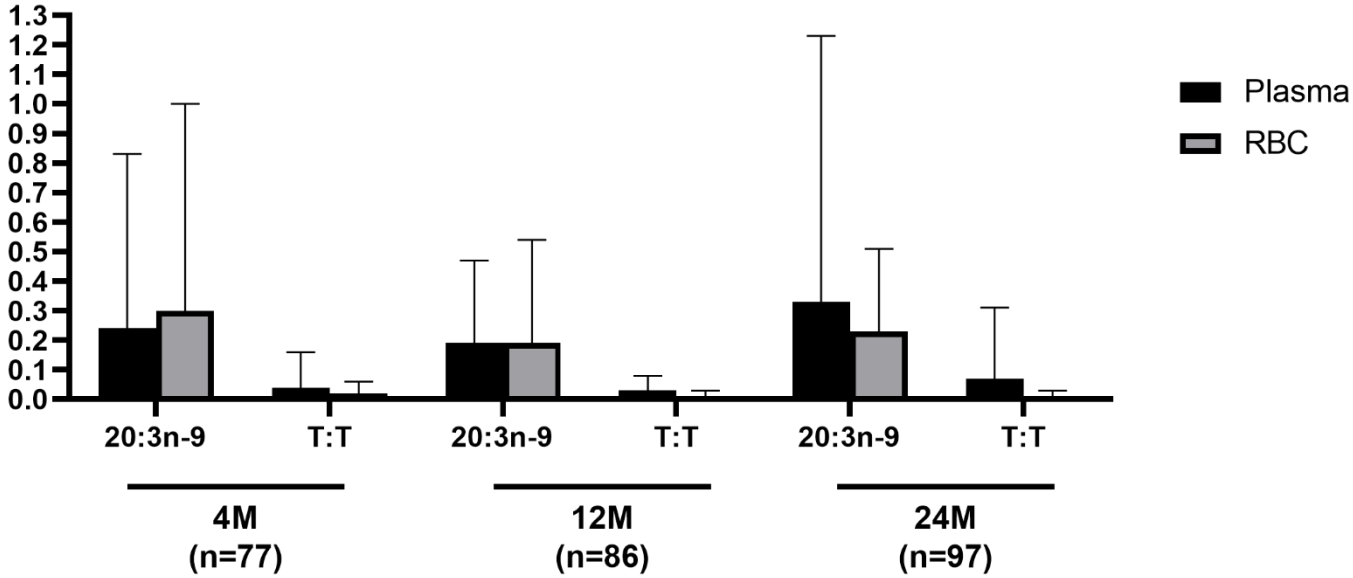


Figure 2. Mead acid and triene:tetraene in plasma and RBC at each time point

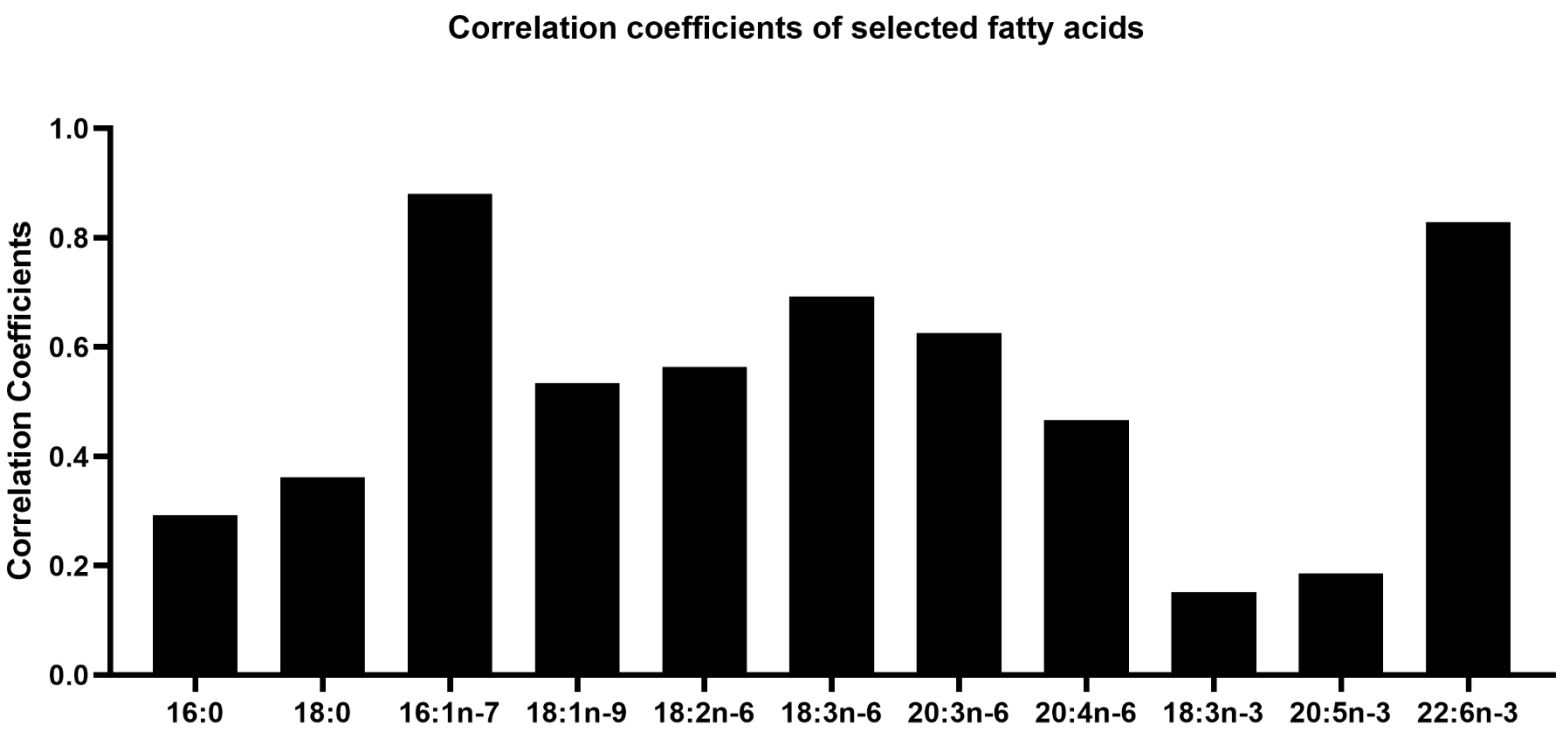


Figure 3. Correlation coefficients between plasma and RBC fatty acid concentrations, n=260, all $p<0.001$ except for ALA (18:3n-3) where $p=0.014$

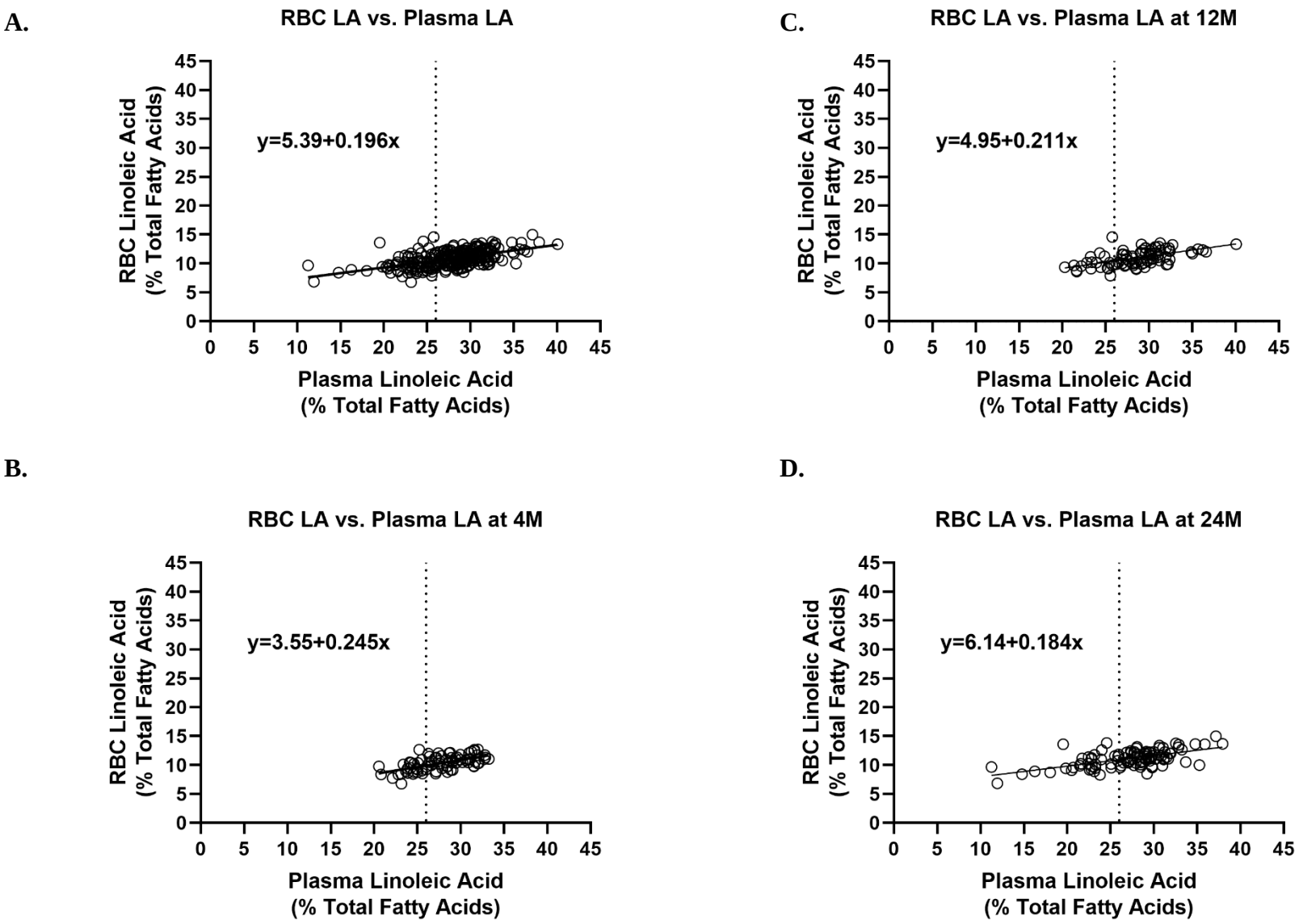


Figure 4. Scatterplots of RBC LA and plasma LA at each time point

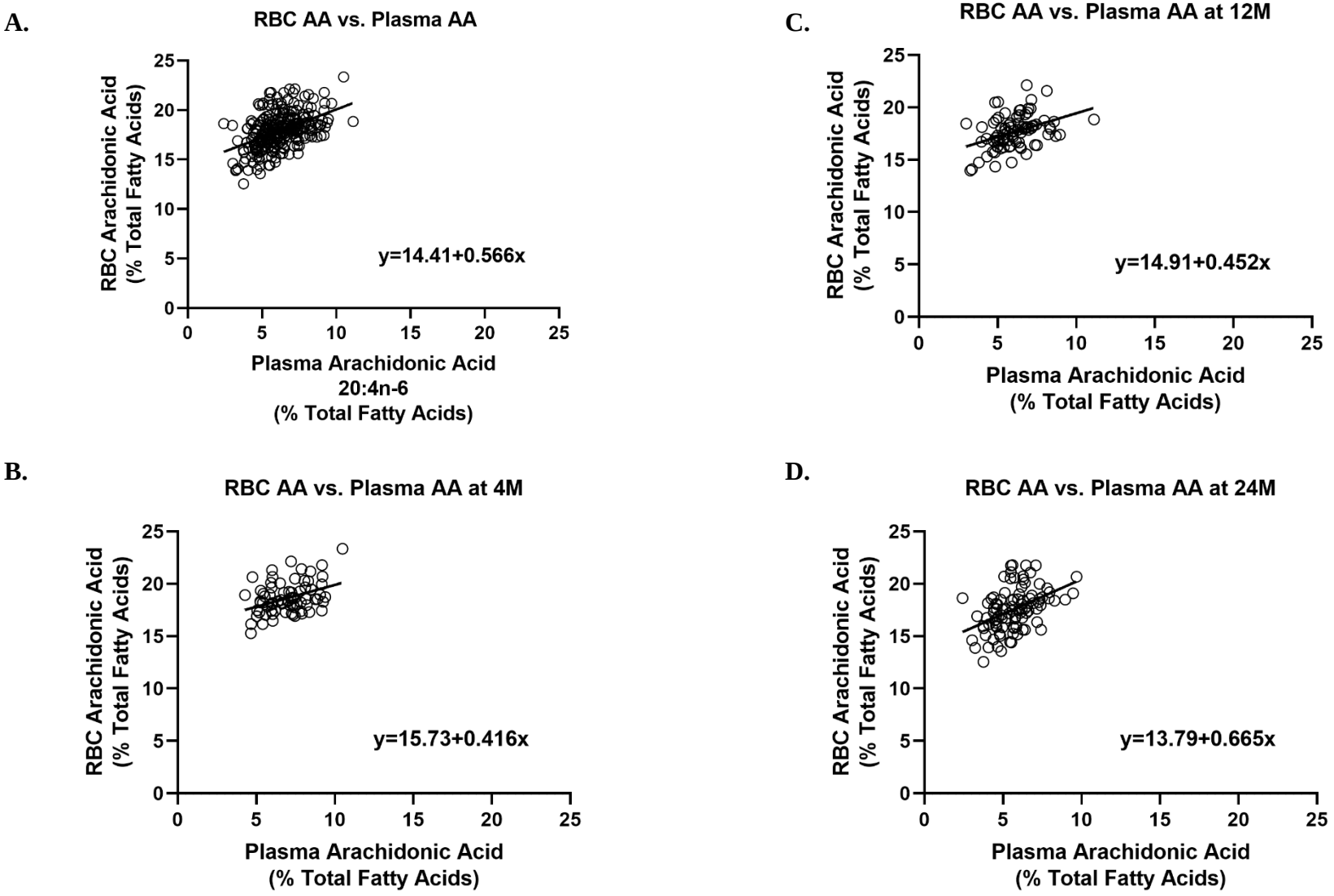
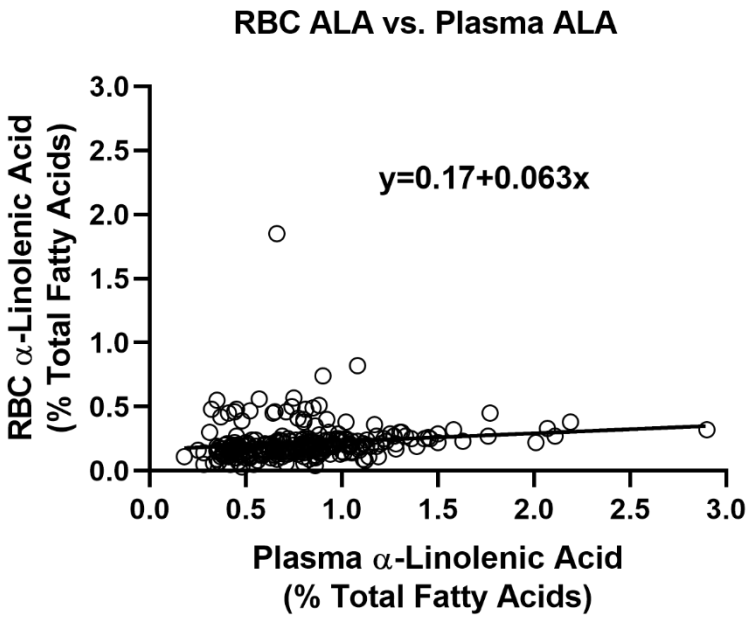


Figure 5. Scatterplots of RBC AA and plasma AA at each time point

A.



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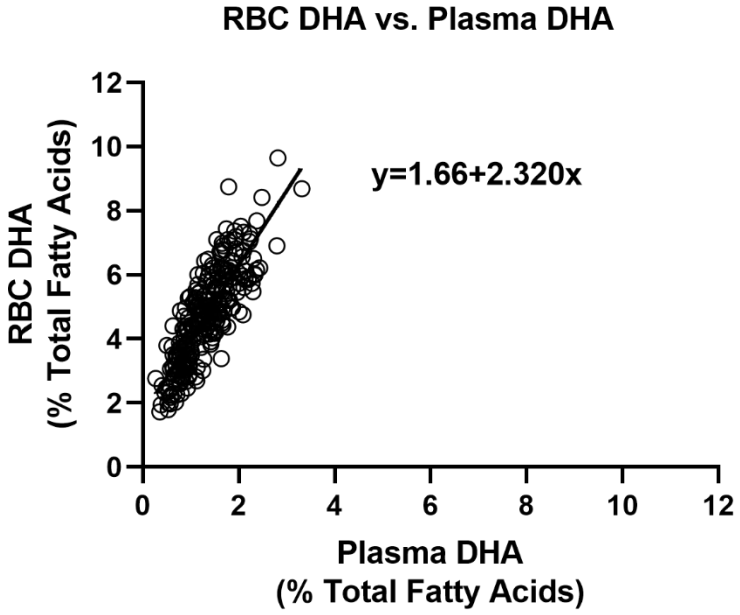

Figure 6. Scatterplots of (A) RBC ALA and plasma ALA and (B) RBC DHA and plasma DHA

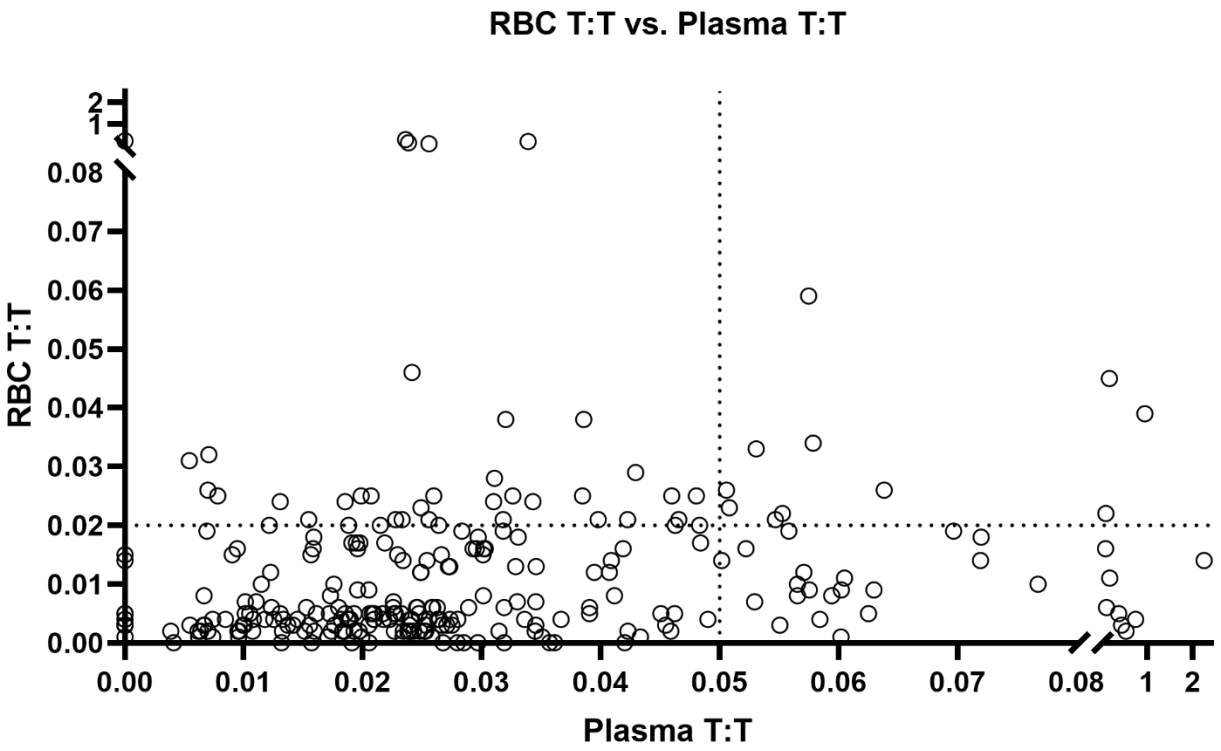

Figure 7. Scatterplot of triene:tetraene in RBC and plasma specimens. RBC T:T of 0.02 is indicative of EFA deficiency whereas plasma T:T of 0.05 is used clinically as the cut-off for EFA deficiency